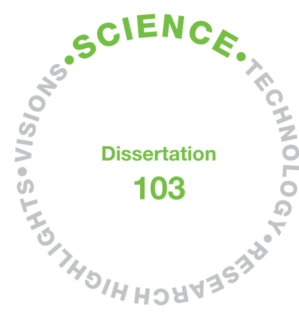
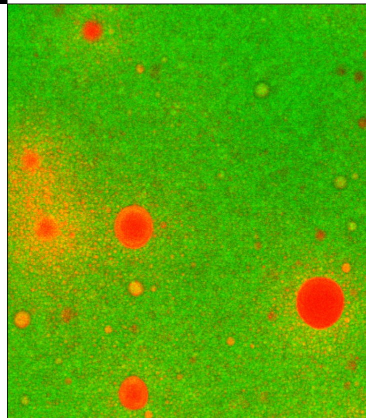


Role of chemical and enzymatic modifications of milk proteins on emulsion stability/properties

Approaches for more stable protein emulsions

Hairan Ma



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Role of chemical and enzymatic modifications of milk proteins on emulsion stability/properties

Approaches for more stable protein emulsions

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Abstract

Milk proteins, sodium caseinate (CN) and whey protein isolate (WPI) are used in food industries as emulsifiers. The stability of an O/W emulsion is dependent on the electrostatic and steric repulsion provided by the interfacial proteins against droplet aggregation or lipid oxidation. Therefore, modifications of the surface charge or the interfacial conformation of protein emulsifiers are expected to enhance their emulsifying properties and emulsion stability. In this present work, sodium caseinate and whey protein isolate were modified by different chemical and enzymatic approaches. The modified proteins were characterized using multiple techniques, and the effect of these modifications on emulsifying properties of proteins and emulsion stability were investigated.

Succinylation converts the positively charged amino groups into negatively charged carboxyl groups, lowering the isoelectric point (pI) of protein. The ethylene diamine (EDA) modification worked in the opposite way, leading to an increased pI. The extent of these two modifications was studied using SDS-PAGE and MALDI-TOF mass spectrometry. The pI of succinylated and EDA modified milk proteins was studied using zeta-potential measurement. As a result, the succinylation to full extent altered the pI of CN from 4.2 to 2.7, and the EDA modification shifted the pI of CN and WPI from 4.2 to 9.4 and from 4.9 to 9.5 respectively. The pH stability of emulsion made with the modified milk proteins was monitored by following the increase of particle size during storage. The results suggested that succinylation and EDA modification could enhanced the emulsion stability at pH 4–7 by increasing the electrostatic repulsion between droplets.

Regarding the enzymatic modification of milk proteins, the laccase and transglutaminase (Tgase) catalyzed cross-linking were applied on WPI and CN respectively. In order to improve the reactivity of WPI towards the laccase, a vanillic acid modification was carried out to incorporate additional methoxyphenol groups into the protein surface. The cross-linking of vanillic acid modified WPI (Van-WPI) by laccase was studied using SDS-PAGE. The extent of cross-linking of Van-WPI was found to be significantly higher compared to the unmodified WPI and the combination of WPI and free phenolic compound as a mediator. The effect of laccase catalyzed cross-linking on storage stability was investigated by visual observation and confocal microscopy. The post-emulsification cross-linking was

proven to enhance the stability of the emulsions prepared with Van-WPI during the storage. The reduced droplet coalescence could be most likely attributed to an extended interfacial protein layer formed via the interaction between the adsorbed proteins and non-adsorbed proteins in the water phase. In contrast with the limited extent of cross-linking of WPI by laccase, CN was extensively cross-linked by Tgase. The physical stability of emulsions was studied by measuring the increase of particle size during storage, and the oxidative stability was evaluated by following the formation of fatty acid hydroperoxides and volatile compounds in different stages of the lipid oxidation. The pre-emulsification cross-linking showed no obvious influence on the physical stability of CN emulsion but significantly improved its stability against lipid oxidation. The improvement of oxidative stability of emulsions could be contributed to a thicker and denser interfacial protein layer and thus increases the amount of anti-oxidative groups located at the interface and provides a stronger barrier against competitive adsorption by oil oxidation products.

Preface

The study described in this thesis was carried out at VTT Technical Research Centre of Finland during the years 2007–2014. The research was carried out in the Marie Curie EU project Pro-Enz (Enzymatic tailoring of polymer interactions in food matrix) and the project “Novel Protein-Based Emulsion by Engineering Interfacial Mass Transfer”. The Graduate School for Biomass Refining of the University of Helsinki, the Academy of Finland and the Finnish Cultural Foundation are acknowledged for partially funding this research.

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Hangzhou, June 2015,
Hairan

Academic dissertation

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List of publications

This thesis is based on the following original publications which are referred to in the text as I–IV.The publications are reproduced with kind permission from the publishers.

I Ma, H., Forssell, P., Partanen, R., Seppänen, R., Buchert, J., Boer, H. 2009. Sodium caseinates with an altered isoelectric point as emulsifiers in oil/water systems. Journal of Agricultural and Food Chemistry, *57,* 3800–3807.

II Ma, H., Forssell, P., Partanen, R., Buchert, J., Boer, H. 2011. Charge modifications to improve the emulsifying properties of whey protein isolate. Journal of Agricultural and Food Chemistry, 59, 13246–13253.

III Ma, H., Forssell, P., Partanen, R., Buchert, J., Boer, H. 2011. Improving laccase catalyzed cross-linking of whey protein isolate and their application as emulsifiers. Journal of Agricultural and Food Chemistry, 59, 1406–1414.

IV Ma, H., Forssell, P., Kylli, P., Lampi, A.-M., Buchert, J., Boer, H., Partanen, R. 2012. Transglutaminase catalyzed cross-linking of sodium caseinate improves oxidative stability of flaxseed oil emulsion. Journal of Agricultural and Food Chemistry, 60, 6223–6229.

Author's contributions

I. The author planned the work together with Prof. Johanna Buchert, Dr. Harry Boer, Dr. Pirkko Forssell and Dr. Riitta Partanen. The author conducted the laboratory work and was responsible for the data analysis. Part of the experimental work was performed at Institute for Surface Chemistry in Sweden under the guidance of Dr. Rauni Seppänen. The author wrote the paper under the guidance of Dr. Harry Boer.

II. The author planned the work together with Prof. Johanna Buchert, Dr. Harry Boer, Dr. Pirkko Forssell and Dr. Riitta Partanen. The author conducted the laboratory work and was responsible for the data analysis. The author wrote the paper under the guidance of Dr. Harry Boer.

III. The author planned the work together with Prof. Johanna Buchert, Dr. Harry Boer, Dr. Pirkko Forssell and Dr. Riitta Partanen. The author conducted the laboratory work and was responsible for the data analysis. The author wrote the paper under the guidance of Dr. Harry Boer.

IV. The author planned the work together with Prof. Johanna Buchert, Dr. Harry Boer, Dr. Pirkko Forssell and Dr. Riitta Partanen. The author conducted the experiments concerning the oxidative stability of emulsions. The gas chromatography analysis was performed at the University of Helsinki with Dr. Petri Kylli under the guidance of Dr. Anna-Maija Lampi. The author wrote the paper under the guidance of Dr. Riitta Partanen.

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List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AFM	atomic force microscopy
BSA	bovine serum albumin
CN	sodium caseinate
CLSM	confocal laser scanning microscopy
DH	degree of hydrolysis
EA	emulsifying activity
EAI	emulsifying activity index
EC	emulsifying capacity
EDA	ethylene diamine
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ES	emulsion stability
ESI	emulsion stability index
GC	gas chromatography
HLB	hydrophilic-lipophilic balance
HLPC	high-performance liquid chromatography
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MDA	malondiadehyde
MS	mass spectrometry
M_w	molecular weight
NMR	nuclear magnetic resonance
O/W	oil-in-water

pI	isoelectric point
PV	peroxide value
SDS-PAGE	sodium docecyl sulfate polyacrylamide gel electrophoresis
Sulfo-NHS	N-hydroxysulfosuccinimide sodium
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
Tgase	transglutaminase
TNBSA	2,4,6-trinitro-benzene sulfonic acid
UV	ultra-violet
W/O	water-in-oil
WPI	whey protein isolate

1. Introduction

1.1 O/W emulsions and emulsifiers

Oil-in-water (O/W) emulsions are mixtures containing oil as dispersed phase and water as dispersion medium. The two immiscible liquids in emulsion are often mixed by high external energy, for example high pressure or high speed blending, and the oil phase eventually breaks into small droplets. O/W emulsions are used in various industrial fields such as foods, cosmetics, lubricants and pharmaceuticals for the transfer, delivery or controlled release of active components [Gallegos & Franco 1999]. Different types of ingredients, for example emulsifiers, salts, colorants, antioxidants and hydrocolloids, can be added to emulsions with subsequent functional contribution to the sensory and stability of an emulsion-based product [McClements 2004].

Emulsifiers are used in emulsions to facilitate the homogenization process and assist in maintaining the emulsion stability (ES) due to their amphiphlic nature. Addition of emulsifiers reduces the interfacial tension between the oil and water phases, decreasing the energy needed to disrupt the droplets during the homogenization [Dickinson 1993]. Due to the immiscible nature and different density of oil and water, emulsions are thermodynamically unstable and phase separation tends to take place during storage. Emulsifiers form a protective shell around the droplets generated in the emulsification process. This layer in turn may prevent the droplets from aggregation and retard the mass transfer between the two phases [Capek 2004]. Large molecular weight (Mw) emulsifiers such as proteins or other amphiphilic polymers can stabilize the interface by forming an immobile viscoelastic film, while an interfacial layer built by small molecular surfactants is highly mobile [Bos & van Vliet 2001, Tcholakova et al. 2008]. The formation of an O/W emulsion is presented in Fig. 1.

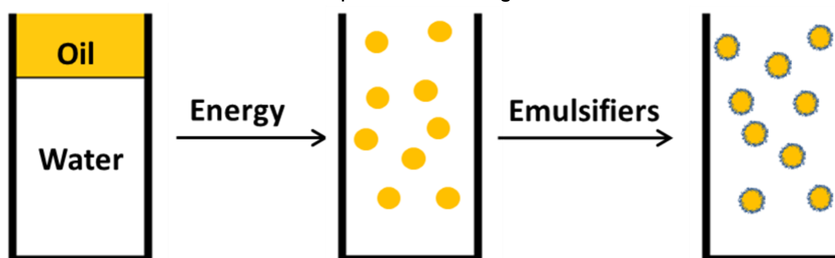


Figure 1. Formation of emulsions with assistance of emulsifiers.

1.2 Milk proteins and their role as emulsifiers

Milk proteins have high nutritional value, excellent surface properties and high sensory characteristics [Dickinson 1997]. The two main classes of milk proteins are caseins and whey proteins. The casein family, including α_{s1}-, α_{s2}-, β-, and κ-casein, represents approximately 80% of the total weight of cow's milk proteins. Casein monomers exhibit flexible random coil structures while in their natural state in milk they tend to aggregate into a micellar form [Walstra 1999]. Whey proteins, including α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA) and immunoglobulins, represent the residual 20% of milk proteins and are distinguished from caseins mainly for the stability around their isoelectric points (pI) and rigid globular structures [Goff & Hill 1993]. Both caseins and whey proteins have been widely used as food emulsifiers due to their excellent emulsifying capabilities [Dickinson 1998a]. The hydrophobic moieties of these milk proteins can readily adsorb onto the surface of newly created oil droplets during the emulsification procedure and the hydrophilic parts stretch out in the water phase, forming a protective layer at the interface. This interfacial layer provides an electrostatic repulsive force against droplet aggregation since the surface proteins are of the same type of charge. At the same time the protruding hydrophilic groups build a steric hindrance against a direct contact between the cores of droplets during collision [Stauffer 1999]. Caseins have a relatively high surface activity and can rapidly decrease the surface tension attributed to their open structures. Therefore they are believed to have higher emulsifying activities than whey proteins due to a higher rate of adsorption at the oil and water interface [Sharma & Dalgleish 1993]. The thickness of the adsorbed layer of caseins on a solid surface is around 10 nm [Husband et al. 1997]. For globular whey proteins, it takes longer time for the adsorption and the conformational rearrangement at the O/W interface during and after the emulsification stage. However, once the interfacial films are formed, the interfacial whey proteins are believed to provide better surface elasticity against coalescence than caseins [Boyd et al. 1973]. O/W interface stabilized by caseins, whey proteins and small molecular surfactants are presented in Fig. 2.

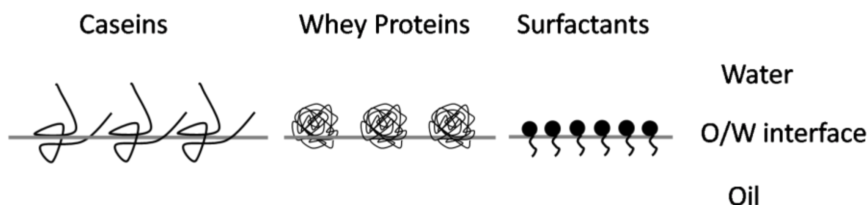


Figure 2. Adsorption of caseins, whey proteins and surfactants at O/W interface.

1.3 Emulsion stability

Emulsion stability describes the resistance of an emulsion against physical and chemical changes over time. In general, emulsion stability largely depends on the production, storage conditions and the physicochemical properties of its compositions. A good understanding of how emulsion stability is affected by the relevant factors such as the structure and charge of the interfacial layer and the surrounding molecular environment is the base for predicting and improving the quality of an emulsion product.

1.3.1 Physical stability of emulsions

The thermodynamic stability of emulsions depends on the free energy of the O/W systems before and after emulsification [Hunter 1989]. In general, formation of macroemulsions which refer to those containing droplets with average diameter over 0.1 μm is thermodynamically unfavorable due to the increase of interfacial area. Microemulsions with extremely small interfacial tension and droplets size (diameter less than 0.1 μm) are thermodynamically stable if the increased interfacial energy can be overcome by the increase of configurational entropy [Solans et al. 2005]. In the scope of this work, only the instability of macroemulsions is concerned.

Destabilization of emulsions often leads to physical changes in the spatial distribution or structural organization of droplets. The most common destabilization behaviors in an O/W emulsion are creaming, flocculation and coalescence [McClements 2004]. Creaming occurs due to the gravitational difference between oil and water. The oil droplets move upwards and gradually form a dense layer at the top of the emulsion [Robins et al. 2002]. Flocculation refers to the droplets aggregation in which the droplets stay together after collision with their intact identities [Dickinson 1998b]. Coalescence describes another type of aggregation whereby the droplets merge into larger droplets after collision [Tcholakova et al. 2006]. Other destabilization phenomena of importance include Ostwald ripening and phase inversion. In Ostwald ripening large droplets grow at the expense of smaller ones due to the diffusion of molecules from small droplets to larger droplets upon dissolution in the continuous phase [Taylor 1998]. During phase inversion O/W type emulsion changes into water-in-oil (W/O) type or vice versa as a result of the spontaneous rearrangement of interfacial surfactants [Araújo & de Oliveira 2011]. The destabilization process, if properly controlled, can sometimes assist the formulation of emulsion products or help to improve their quality. For example, the phase inversion process is a crucial step in butter and margarine production, and a controlled extent of flocculation creates desirable texture by increasing the viscosity of products [McClements 2010]. However, in most cases the destabilization causes undesirable effects and may substantially impair the appearance, texture, taste and stability of emulsions.

A number of factors are decisive for the physical stability of an emulsion as they influence the creaming velocity and the collision frequency. The creaming velocity of an O/W emulsion is dependent on the size of the oil droplets, the density difference between the dispersed and continuous phases, and the viscosity of continuous phase, as described for a single droplet by the Stoke's law (1):

$$v = -2gr^2 (\rho_2-\rho_1)/9\eta_1 \quad (1)$$

where v is the creaming velocity, g is the gravitational acceleration, r is the radius of oil droplets, ρ_2 and ρ_1 are respectively the density of dispersed and continuous phases, and η_1 is the viscosity of continuous phase.

One way to retard the creaming process is by minimizing the density difference between dispersed phase and continuous phase. Often in flavor industry, an oil phase with a density near to the water phase can be prepared by dissolving weighting agents which have higher density than water into the oil phase [Chanamai & McClements 2000]. However, manipulating the density difference is not suitable for applications where the oil type or the oil composition is restricted. A more practical approach for controlling the creaming process is to reduce the droplet size by increasing the energy input during homogenization or to increase the viscosity of the continuous phase using thickening agents [Schultz et al. 2004, Dickinson 2009].

Collision between oil droplets causes their subsequent flocculation or coalescence behavior. The extent of droplet aggregation largely depends on the frequency and efficiency of the collisions. A low oil volume fraction, large droplet size and high viscosity can reduce the collision frequency of oil droplets and a strong interparticle repulsive force prevents droplet aggregation during collisions. In the case of a protein stabilized O/W emulsion, a thick interfacial protein film can provide steric and electrostatic repulsion between the oil droplets, preventing colliding droplet surfaces from getting close to each other. Temperature, pH and ionic strength are also important factors that affect emulsion stabilities since they could significantly influence the droplet mobility and the interaction between droplets [Neirynck et al. 2007, Losada-Barreiro et al. 2012, Wu et al. 2012].

1.3.2 pH stability of emulsions

Emulsion stabilized by ionic emulsifiers are very sensitive to the pH changes since the electrostatic repulsion between droplets is pH-dependent and the charge of the emulsifier can greatly influence the interaction between the oil droplets and other components in an emulsion. For a protein stabilized emulsion, the strength of electrostatic repulsion between droplets depends on the magnitude of the interfacial protein. Protein carries more positive or negative charge at a pH far away from its pI and provides stronger repulsive force against droplet aggregation than it does as the pH approaches its pI [Damodaran 1996]. Besides the magnitude, the cationic or anionic character of interfacial proteins also influence the emulsion stability since it is decisive for an attractive or repulsive interaction between the protein and other emulsion stabilizer such as polysaccharides. For example, some acidic food emulsions are prepared with protein and pectin. At acidic pH below the protein's pI where the protein carries positive charge, the interfacial protein can conjugate with the negatively charge pectin to form a thicker interfacial layer, leading to an improved emulsion stability [Neirynck et al. 2004].

1.3.3 Oxidative stability of emulsions

Besides the physical aspects, chemical stability of emulsions is also a concern. The most common chemical reaction in an O/W emulsion is lipid oxidation which is a free radical chain reaction and develops a number of adverse effects such as “off-flavors” and potential toxicity [Kubow 1992]. The lipid oxidation involves a complex series of radical chain reactions between lipids and active oxygen species. The oxidation is considered to occur in three different stages: initiation, propagation, and termination [Nawar 1996]. Since the formation of free radicals via the reaction between fatty acid and oxygen is thermodynamically difficult, the initiation of lipid oxidation was postulated to take place by hydroperoxides decomposition. In the initiation stage, the lipid hydroperoxides at the droplet surface are broken down into highly reactive peroxyl (2) and alkoxyl radicals (3) by transition metals or pro-oxidants. These radicals react with unsaturated lipids, forming free radicals which can be further converted into peroxyl radicals with free oxygen (4, 5 and 6). During the propagation stage, the lipid peroxyl radicals abstract hydrogen from another fatty acid to produce hydroperoxides and free radicals (7). This reaction proceeds until there is no hydrogen source available or the chain is interrupted. The oxidation is terminated when radicals react with each other, forming polymers and nonradical monomer products including aldehydes, ketones, alcohols and alkanes which dramatically change the physicochemical and sensory properties of oils (8).

$$ROOH \rightarrow ROO^{\bullet} \quad (2)$$

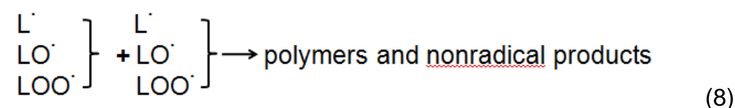
$$ROOH \rightarrow RO^{\bullet} \quad (3)$$

$$ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet} \quad (4)$$

$$RO^{\bullet} + RH \rightarrow ROH + L^{\bullet} \quad (5)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (6)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (7)$$



$$\left.\begin{matrix} L^{\cdot} \\ LO^{\cdot} \\ LOO^{\cdot} \end{matrix}\right\} + \left.\begin{matrix} L^{\cdot} \\ LO^{\cdot} \\ LOO^{\cdot} \end{matrix}\right\} \rightarrow \text{polymers and nonradical products} \quad (8)$$

Oxidative stability of emulsions can be improved by using saturated lipids instead of unsaturated ones since they are more stable against oxidation. However, the use of unsaturated lipids in emulsions is highly desired due to their better nutritional properties. Therefore emulsion manufacturers have attempted to limit

lipid oxidation by other means such as excluding oxygen from the system, reducing hydroperoxides, transition metals, pro-oxidants in emulsion ingredients, and adding antioxidants [McClements & Decker 2000].

Proteins are emulsifiers with intrinsic antioxidant properties due to their capability to inactivate reactive oxygen species, scavenge free radicals and chelate transition metals [Elias et al. 2008]. For protein-stabilized emulsions, efforts have been made to further retard oxidation by modifying some of the droplet characteristics such as the droplet size, surface charge, and thickness of the interfacial proteins. There seems, however, to be a great confusion concerning how the oxidation rate is affected by droplet size. Osborn and Akoh [2004] reported that the oxidation rate in a lipid-based O/W emulsion is independent with droplet size. Some studies reported an increasing rate of oxidation with smaller droplet size [Nakaya et al. 2005, Imai et al. 2008]. On the contrary, less extensive oxidation was found in emulsions with larger droplets since at a fixed total volume of the oil phase, a smaller surface area of droplets leads to less contact between oxygen and lipids [Gohtani et al. 1999]. This approach, however, does not seem to be very feasible since increased droplet size associates with physical instability of emulsion.

Surface charge of droplets can significantly influence the interaction between the oil phase and the aqueous phase. A cationic surface of the droplet can repel metal ions (e.g. Fe, Co, Cu) and other positively charged pro-oxidants in the aqueous phase and thus effectively limit the lipid oxidation [Donnelly et al. 1998], and whereas emulsions stabilized by anionic emulsifiers were found to be oxidized more quickly [Mancuso et al. 1999]. For this reason, protein emulsifiers were suggested to be used at pH values below their iso-electric points where the surface proteins carry a net positive charge [Donnelly et al. 1998]. Another practical scenario is to increase the thickness of the interfacial protein layer. A thicker protein layer on droplet surface would build a stronger mechanical barrier between the lipid substance and the pro-oxidants in the aqueous phase and thus retard lipid oxidation [Hu et al. 2003].

1.4 Characterization of a protein-stabilized emulsion

A protein covered emulsion droplet can be characterized by various techniques to evaluate the performance of protein emulsifiers and the stability of emulsion. The structure, strength and charge of surface proteins is generally reflected in the resistance of emulsion droplets against aggregation and chemical changes, and therefore is associated with the physical and chemical stability of an emulsion. The size and migration of droplets during the storage of emulsions can be used as an indication of the physical emulsion stability. Inside the oil droplets, the products of lipid oxidation are normally determined as an approach to study the oxidative stability.

1.4.1 Emulsifying efficiency of proteins

Commonly considered parameters for evaluating the performance of emulsifiers are emulsifying capacity (EC), emulsifying activity index (EAI) and emulsion stability index (ESI). EC is defined as the maximum amount of oil that can be emulsified in an aqueous solution containing a unit mass of emulsifier [Swift et al. 1961]. It is normally determined by titrating small volume of oil into an aqueous protein solution during the homogenization by high speed blending. The titration reaches the end point when the emulsion breaks down or phase inversion takes place. Several experimental methods such as optical and electrical conductivity measurements can be used to indicate the end point of titration [Crenwelge et al. 1974, Marshall et al. 1975, Pearce & Kinsella 1978]. EAI refers to the interfacial area stabilized by unit amount of emulsifier. According to the Mie Theory for light scattering, the interfacial area can be estimated by turbidimetric methods that measure the scattered light at an angle of 90 degree, and with a known oil volume fraction and protein concentration in the aqueous phase, the EAI is defined as (9):

$$EAI = 2T/\phi C \qquad (9)$$

where T is the turbidity of the emulsion sample at a wavelength of 500 nm, ϕ is the volume fraction of the oil phase, C is the protein concentration in the aqueous phase before emulsification [Pearce & Kinsella 1978].

The ESI is a measure of the half life of an emulsion. It can be expressed as the time at which the turbidity of an emulsion reduces to half of its original value [Kim et al. 2004]. The measurements of EAI and ESI, however, are based on the assumption that the droplets are spherical and large enough compared to the wavelength of the scattered light. In many cases, the size of droplets is of the same order of magnitude as the light wavelength, which would cause experimental errors. Another limit of the use of EAI and ESI is that they do not give any direct information about the droplet size which is of utmost importance for an emulsion. Therefore, the initial droplet size of freshly made emulsion is more often used as an indication of the EA of an emulsifier.

1.4.2 Droplets characterization

The characterization of droplets in an O/W emulsion mainly includes particle sizing, determination of droplet charge and monitoring of droplet behavior during storage (creaming and aggregation). The advantages and disadvantages of some commonly used techniques are summarized in Table 1. Zeta-potential measurement is particularly important for this work where the effect of interfacial charge on the pH stability of protein stabilized emulsion is studied. The liquid layer surrounding a particle can be divided into an inner region (Stern layer) where the ions move together with the particle due to a strong binding, and an outer region in which a boundary between the ions move with and without the particle can be drawn. The zeta-potential is a measure of the potential at this boundary (slipping plane or hydrodynamic shear). Emulsions with strong positive or negative zeta potential (more positive than +30 mV or more negative than -30 mV) own a higher possibility to be stable since the droplets repel each other and have less tendency to aggregate [Hunter 1989].

Table 1. Techniques to characterize emulsion droplets.

Methods	Advantages	Disadvantages	References
Light Microscopy	Simple sample preparation Distinguishes between flocculation and coalescence	Cannot detect small droplets under 1 μm The structure of specimen might be altered by sample preparation	[Ferrando & Spiess 2000]
Electron Microscopy	Imaging of smaller droplets above 1 nm, protein aggregates and interfacial films 3D structure can be generated by SEM	Demanding sample preparation Possible artifacts emerge during sample preparation Expensive instruments Only for dry or slurry samples	[Smart et al. 1995]
Atomic Force Microscopy (AFM)	Imaging of small emulsion components at atomic and molecular levels Imaging the structure of interfacial protein membrane	Demanding sample preparation Only for dry or slurry samples	[Kirby et al. 1995]
Light Scattering	Wide range of droplet size (0.1-1000 μm) can be detected Determination of the particle size	Difficult to distinguish between flocculation and coalescence Dilution might disrupt flocculated	[Rysakov & Rejmund 2008]

	distribution and calculation of surface area	droplets Large variation between instruments made by different manufacturers	
Nuclear magnetic res onance (NMR)	Sensitive to particle size between 0.2 to 100 µm Independent of droplet flocculation Non-disruptive sample preparation Suitable for concentrated emulsions	Expensive instrument Complicated data processing	[Dickinson & McClements 1995, McClements 2004]
Electroacoustics	Analyzes droplets with size between 0.1 to 10 µm Suitable for concentrated emulsions Determine both size and zeta-potential	Limited to charged droplets in conductive medium Demands significant density difference between the oil phase and the aqueous phase	[McClements 2004, Coupland & McClements 2001]
Zetasizer	Measures the zeta-potential and particle size	Dilution of sample often needed	[Hunter 1989]

1.4.3 Interfacial properties of proteins

Interfacial tension and rheology are other important aspects in emulsion formation and emulsion stability. Addition of proteins into the aqueous phase lowers the interfacial tension between oil and water phases and may thus reduce the energy needed for the emulsification process. Static measurement of the interfacial tension at an interface which has already reached equilibrium can be carried out using several methods such as the Du Nouy ring, Wilhelmy plate or sessile and pendant drop. Dynamic interfacial tension can be measured to follow the adsorption kinetics of proteins using a Wilhelmy plate or pendant drop methods [Couper 1993]. The interfacial rheological characteristics of proteins play an important role for emulsion stability. The adsorbed proteins, depending on the adsorption kinetics and surface concentration, may unfold and interact with their neighboring molecules, resulting in a two dimensional viscoelastic gel which provides resistance to the deformation of the interface caused by external disturbance [Murray 2002, Wierenga et al. 2006]. Various types of rheometers have been developed to study the response of interfacial proteins under shear or dilatational stress [Miller et al. 2010]. These studies could provide valuable information about the adsorption kinetics, structure of the interfacial layer and interactions of proteins at the interface.

1.4.4 Lipid oxidation

The lipid oxidation in an emulsion can be assessed by a number of analytical methods that measure the loss of initial reactants or the formation of oxidation products. The oxygen consumption can be monitored by an oxygen meter, and the reduction of oxidazable lipids or the formation of oxidation products can be measured by gas chromatography (GC) or high-performance liquid chromatography [Coupland & McClements 1996]. Various types of techniques can be used to quantify lipid oxidation products such as hydroperoxides. Commonly used methods include high-performance liquid chromatography (HPLC), spectrophotometric assay for conjugated dienes at 234 nm and iodometric titration peroxide value (PV) methods [Paquot & Hautfenne 1987, Steger & Mühlebach 1998, Henderson et al. 1999]. Ultra-violet (UV) spectroscopy is also useful to measure some of the secondary products of the oxidation. The most commonly used method is the Thiobarbituric acid reactive substances (TBARS) assay which is based on the reaction between malondiadehyde (MDA) and thiobarbituric acid (TBA) reagent. The reaction yields a chromophore absorbs at 532 nm to give pink color [McDonald & Hultin 1987]. Volatile aldehydes formed during the termination stage of lipid oxidation, for example propanal and hexanal, are often determined using head space gas chromatography analysis [Stevenson et al. 1996].

1.5 Modification of the emulsifying properties of proteins

Based on the theories on formation and stabilization of protein emulsions, various modifications have been carried out to improve the emulsifying properties of proteins. The majority of these efforts have focused on enhancing the adsorption of proteins onto the O/W water interface, increasing the interfacial charge, altering the conformation of the interfacial layer and strengthening the interaction with other emulsion components such as polysaccharides. The modifications are summarized below.

1.5.1 Physical modifications

Adjustment of physical conditions such as temperature, pH, ionic strength or addition of ingredients which interact with proteins can be applied before or during the emulsification process to improve the formation of emulsions and their stability [Tcholakova et al. 2006]. Heat treatment of proteins prior to emulsification can often induce conformational changes in proteins. If properly controlled, it can increase the surface hydrophobicity of proteins as a result of exposure of buried hydrophobic groups [Nakai & Li-Chan 1988]. A more hydrophobic surface would facilitate the adsorption of proteins onto interface and thereby improve their emulsifying activity (EA) [Moro et al. 2001]. pH and ionic strength significantly influence the charge of interfacial proteins. Desirable EA of whey, chickpea and soybean proteins has been obtained by preparing the aqueous phase at pH values far away from their pI at minimized salt concentration [Mitidieri & Wagner 2002, Zhang et al. 2009]. Addition of other functional components into the aqueous phase may also assist the emulsion formation or help to maintain stability by changing the protein structure or interacting with proteins. For example, casein micelle, an aggregate consisting of α-casein and β- casein and stabilized by an outer hairy layer of κ-casein, dissociates when sodium citrate was added to induce complexation of calcium. As a result EA was improved since the protein more efficiently adsorbs onto the interface in a dissociated state [Shirashoji et al. 2006]. An interfacial multilayer could be formed by addition of anionic polysaccharide into a protein stabilized emulsion at a proper pH, leading to a thicker interfacial film and improved stability against droplet aggregation [Surh et al. 2006].

1.5.2 Chemical modifications

Protein modifications using chemical agents are in many cases not suitable for food related applications due to safety concerns. However, chemical modifications have been widely studied to demonstrate how specific functionalities of proteins affect the emulsion properties. Based on the impact brought by modifications, the chemical reactions are divided into two groups: i), reactions which alter the pI of proteins; ii), reactions which alter the structure of interfacial protein layer.

Chemical reactions that take place on the amino groups, for example acylation and the Maillard reaction, can reduce the positive charge of a protein when the positively charged amino groups are be substituted by neutral or negatively charge ones and thereby lowering the pI of the protein [Lawal & Adebowale 2004, Oliver et al. 2006]. Reactions such as esterification in which negatively charged carboxyl groups of protein are abolished shift the pI of proteins towards the alkaline region [Sitohy et al. 2001]. Other chemical modifications can also affect the charge of proteins by adding charged side groups. For example, deamidation converts the non-charged amide groups to negatively charged carboxyl groups and phosphorylation adds phosphate groups with negative charge, resulting in a lowered pI [Van Hekken et al. 1996, Flores et al. 2010]. Modifications using chemical agents with cationic side groups could raise the pI of proteins [Hattori et al. 2000].

Another group of modifications incorporate moieties into the proteins to enable them to adsorb onto the interface with a conformation which is favorable for the formation or stability of emulsions. For example, thiolation incorporates extra thiol groups which further strengthen the interfacial protein layer by forming disulfide bonds [Stevenson et al. 1997]. Acylation adds an alkyl group into the protein structure with increased surface hydrophobicity that favors the adsorption of proteins onto the interface during emulsification [Lawal & Adebowale 2004, Matemu et al. 2011]. A Maillard reaction using oligosaccharides or polysaccharides leads to a higher thickness of the interfacial protein layer which fortifies the emulsions droplets against aggregation [Wooster & Augustin 2006]. Different modifications and their impacts on the emulsifying properties of proteins are summarized in Table 2.

Table 2. Effect of chemical modifications on emulsifying properties of different proteins.

Modifications	Proteins	Modifying agents	Impacts	References
Maillard reaction (Glycation)	β-Lactoglobulin	cationic saccharides	Improved EA; suppressed aggregation at acidic pH conditions	[Hattori et al. 2000]
	β-Lactoglobulin	Dextrans	Improved stability against flocculation	[Wooster & Augustin 2006]
	Casein	Dextran	Improved heat stability, EA and ES	[Aminlari et al. 2005]
	Caseinate	Monosaccharides, disaccharides and oligosaccharide	Similar emulsifying properties to the unmodified caseinate; increased viscosity in the aqueous phase	[Oliver et al. 2006]
	Milk proteins	Glucose and oligosaccharides	Improved emulsifying properties; enhanced antioxidant activity	[Augustin et al. 2006]
Deamidation	Wheat protein		Improved stability against coalescence and heat treatment	[Day et al. 2009]
	Gluten		Improved EA and ES	[Flores et al. 2010]
Thiolation	β-casein	N-acetylhomocysteinthiol	Increased resistance against displacement by surfactant	[Stevenson et al. 1997]

		actone		
Acylation	Mucuna bean protein concentrate	Acetic anhydride and succinic anhydride	Improved EA	[Lawal & Adebowale 2004]
	Soy proteins	Saturated fatty acids	Improved EA and ES	[Matemu et al. 2011]
Phosphorylation	Casein	Phosphorus oxychloride	Improved EA; lower ES	[Van Hekken et al. 1996]
	β-Lactoglobulin	Phosphorus oxychloride	Increased viscosity of emulsion	[Woo & Richardson 1983]
Esterification	Milk proteins, methanol	Ethanol and propanol	Improved EA and ES	[Sitohy et al. 2001]
Cross-linking	β-casein	Glutaraldehyde	Improved ES	[Romoscanu & Mezzenga 2005]

1.5.3 Enzymatic modifications

Changes on the protein structure can also be achieved by enzymatic reactions. Proteolytic enzymes decrease the molar mass of the proteins, whereas crosslinking enzymes can either change the chemistry of the amino acid moieties or increase the molar mass [Tavano 2013, Buchert et al. 2010]. Enzymatic reactions can be performed under mild conditions with great specificity. More importantly, as natural products, enzymes are considered as a safer alternative for chemical reagents to modify proteins for food applications.

Hydrolysis decreases the molecular mass of the protein substrates and increases the number of the exposed ionisable and hydrophobic groups [Panyam & Kilara 1996]. Smaller size and more ionization appear to be beneficial for the formation of emulsions since they often result in an improved solubility of proteins especially around their pI and faster diffusion of the proteins to the interfacial region. However, too short peptides resulting from extensive hydrolysis are not able to stabilize emulsions due to their lower efficiency in reducing the surface tension and a reduced affinity with the interface [Turgeon et al. 1991]. The overall effect of hydrolysis on the emulsifying properties of proteins was found to be very dependent on the selection of enzymes, optimization of the degree of hydrolysis (DH) and other conditions of the emulsification process [Agboola & Dalgleish 1996, Singh & Dalgleish 1998, Caessens et al. 1999].

The effects of enzymatic cross-linking on the emulsifying properties of proteins are introduced in details in the following section.

1.6 Cross-linking of proteins

Cross-linking of proteins is one possibility to modify their emulsion behavior. Proteins can be cross-linked enzymatically or non-enzymatically. Different mechanisms of protein cross-linking are described below.

1.6.1 Non-enzymatic cross-linking

Cross-linking of proteins can be induced via certain physical treatments causing formation of inter- or intra-molecular covalent bonds or with chemical reagents as catalysts. The methods and types of cross-linking are summarized in Table 3.

Table 3. Non-enzymatic cross-linking of proteins.

Methods	Cross-linking formed by	References
Heat treatment	Disulfide cross-links Maillard reaction Isopeptide cross-links	[Havea et al. 2009] [Mat Easa et al. 1996] [Singh 1991]
Heat and alkali treatment	Dehydroproteins	[Friedman 1999]
Photo-oxidative conditions	Biaryl cross-links	[Stachel et al. 1996]
Metal-peptide complexes or hydrogen peroxide	Dityrosine cross-links	[Brown et al. 1995]
Chemical cross-linking	Linkage between amine, sulfhydryl and carboxyl groups on proteins	[Uy & Wold 1977]

1.6.2 Enzymatic cross-linking

Enzymatic cross-linking can influence the emulsifying properties of proteins by changing their conformation at the interface and/or in the continuous phase. In terms of EA of proteins, different effects, depending on the extent of the cross-linking reaction and whether the enzymatic reaction is carried out before or after emulsification, have been reported in literature [Sharma et al. 2002, Hinz et al. 2007]. Extensive inter-molecular cross-linking before emulsification compromises the EA of proteins at low concentration. The cross-linked protein polymers take longer time to adsorb and rearrange their conformation at the interface and therefore there will not be sufficient protein molecules to fully cover the newly formed surface and protect the droplets against aggregation [Liu & Damodaran 1999]. With sufficient protein content, the cross-linked proteins exhibit a similar EA as the non-cross-linked ones [Dickinson & Yamamoto 1996, Færgemand et al. 1999]. As far as ES is concerned, improved stability would be expected since a cross-linked protein layer has been reported to better stabilize the O/W interface with higher viscoelasticity than non-cross-linked one [Færgemand et al. 1999]. However, in real emulsion systems, cross-linking has shown both positive and negative impacts on ES, largely depending on the extent of the cross-linking reaction. A limited extent of cross-linking can improve the ES with stronger steric stabilization provided by a thicker and more viscoelastic interfacial layer. Besides this, in an emulsion with high concentration of non-adsorbed protein, cross-linking can increase the viscosity of the continuous phase and thus restrict the creaming and aggregation behaviours of droplets [Færgemand et al. 1998b, Dickinson et al. 1999, Sharma et al. 2002]. On the other hand, extensive cross-linking can impair the ES since the cross-linked protein might lead to a droplet aggregation by participating in the interfacial areas of different oil droplets or reduce the conformational flexibility of interfacial protein molecules [Flanagan et al. 2003, Hiller & Lorenzen 2009]. Less flexibility of surface proteins could weaken the healing of local thinning of interfacial film or result in the fracture of the interfacial film which makes the emulsion more susceptible to coalescence [Færgemand et al. 1998b].

1.6.3 Cross-linking enzymes

A number of enzymes can catalyze the cross-linking reaction of proteins as reviewed by Buchert et al [Buchert et al. 2010]. The only commercially available food-grade cross-linking enzyme is transglutaminase (TGase; EC 2.3.2.13) which catalyzes inter- or intramolecular cross-linking of proteins by acyl-transfer reaction between a γ-carboxyamide group of glutamine residues and a ε-amino group of lysine residues. The reaction leads to the formation of an ε-(γ-glutamyl)lysine isopeptide bond with one molecule of ammonia generated [Folk & Finlayson 1977] as shown in Fig.3

Figure 3. Transglutaminase catalyzed cross-linking of proteins.

The transglutaminase catalyzed cross-linking has been extensively studied and shown to improve several food functionalities i.e. gel formation, foaming, water-holding and emulsifying properties of various protein substrates [Motoki & Seguro 1998].

Laccases (EC 1.10.3.2) are multi-copper enzymes that catalyze the oxidation of various phenolic compounds by a single-electron oxidation mechanism. The enzyme shuttles electron from its substrates to molecular oxygen, resulting in the formation of water and free radicals which can further undergo non-enzymatic reactions including disproportionation, polymerization, hydration and fragmentation [Thurston 1994, Yaropolov et al. 1994]. The laccase-induced cross-linking of proteins is based on the formation of isodityrosine, dityrosine and disulfide bonds [Figueroa-Espinoza et al. 1998, Mattinen et al. 2005].

So far, the main research activities on laccase have focused on pulp and textile processing [Rodríguez Couto & Toca Herrera 2006]. Only few studies have reported the potential use of laccase in food matrices due to the low efficiency of laccase on protein substrates [Færgemand et al. 1998a, Selinheimo et al. 2008b, Ercili Cura et al. 2009]. To improve the extent of cross-linking, small phenolic compounds are often added as mediator or bridging agents. Instead of a direct oxidation of protein by laccase, small phenolic molecules can be more readily oxidized, and then transfer electrons and create radicals in a protein structure. However, the chemical changes in the structure of these phenolic compounds cause simultaneous color formation which could sometimes limit the use of this method [Lantto et al. 2004, Mustafa et al. 2005].

In addition, proteins can also be cross-linked by other types of enzymes such as tyrosinase, peroxidase and sulfhydryl oxidase. Tyrosinase (EC 1.14.18.1) can oxidize tyrosine residues of a protein to quinone which can form cross-links by reacting with lysyl, tyrosyl and cysteinyl residues [Selinheimo et al. 2008]. Peroxidases use H_2O_2 as an electron acceptor to oxidize a variety of substrates including proteins. Radicals formed as a result of the oxidation can further react with other substrates and protein cross-links are formed via covalent bonds between tyrosine, cysteine and lysine residues [Matheis & Whitaker 1984]. Sulfhydryl oxidases (EC 1.8.3.3) catalyze the formation of disulfide bonds between cysteine residues of proteins [Thorpe et al. 2002].

1.6.4 Reactivity of milk proteins toward Tgase and laccase

The reactivity of milk proteins shows significant variability towards cross-linking enzymes, depending on the accessibility of reactive residues in the protein substrates. Caseins have been proven to be good substrates for Tgase since their flexible open structure allows lysine and glutamine residues more accessible to the active site of the enzyme [Monogioudi et al. 2009]. Individual caseins are more susceptible to Tgase than micellar ordered caseins [Bönisch et al. 2004], and the susceptibility of different components of caseins for Tgase decreases in the order of κ-casein >α-casein>β-casein [Tang et al. 2005]. As comparison, whey proteins which are of globular structures are much less reactive towards Tgase [Ercili-Cura et al. 2012]. Under the same condition, α-lactalbumin was found to be more susceptible to Tgase than β-lactoglobulin and BSA [Han & Damodaran 1996].

The cross-linking of milk proteins by laccase has been less studied. However, similar conclusions were made that caseins can be better cross-linked than whey proteins [Hiller & Lorenzen 2009] and the laccase preferably works on α-lactalbumin than β-lactoglobulin [Færgemand et al. 1998a]. Studies of laccase catalyzed cross-linking of protein substrates have suggested that the reactivity of protein in its natural state toward laccase is very limited since high dosage of the enzyme and phenolic compounds as mediators is needed [Ercili Cura et al. 2009, Selinheimo et al. 2008a].

In general, the susceptibility of whey proteins for cross-linking enzymes could be improved by unfolding the protein structure. Several methods such as addition of reducing agents, pretreatments by high pressure or heat and increasing pH were attempted to increase the extent of cross-linking [Ercili-Cura et al. 2012, Jaros et al. 2006].

1.7 Aims of the work

Some studies have indicated the possibility to improve the performance of protein emulsifiers via chemical or enzymatic modifications. Most of these works focused solely on the physical stability of protein stabilized emulsions while the effect of protein modification on the oxidative stability of emulsion is rarely studied. Furthermore, many previous studies were carried out from an application perspective and lacked in-depth information of the modification methods or the modified proteins. The overall aim of this study was to exploit other methods of protein modification and to investigate the effect of chemical and enzymatic modifications on the emulsifying properties of milk proteins, the pH stability and the oxidative stability of the protein-stabilized emulsions. The modifications carried out in this work were expected to improve the electrostatic or steric repulsion of interfacial proteins against physical destabilization or lipid oxidation. Meanwhile, the modified milk proteins were characterized using multiple techniques in order to gain a better understanding on how to control or improve the extent of these modifications.

In particular, the following points were of major interest in this work:

1) Carry out succinylation and ethylene diamine modification of sodium caseinate and whey proteins, and precisely calculate the number of the modified amino acid residues with matrix assisted laser desorption ionization-time of flight (MALDI-TOF) technique.

2) Study the effect of succinylation and ethylene diamine modifications on droplet charge and pH stablity in the protein stabilized emulsions.

3) Improve the reactivity of whey proteins towards laccase and study the effect of laccase catalyzed cross-linking on whey protein stabilized emulsions.

4) Study the effect of transglutaminase catalyzed cross-linking on the physical and oxidative stability of sodium caseinate stabilized emulsions.

2. Materials and methods

A summary of the modification and emulsion characterization methods used in this study is presented in this section. Detailed descriptions can be found in the original articles (I–IV).

2.1 Raw materials

Sodium caseinate (CN) was obtained from KasLink Foods (Finland) (Protein 94%, Lactose 0.1%, Fat 1%, Ash 3.5%). Lacprodan ® DI-9212 whey protein Isolate (WPI), which was free of lactose (lactose content below 0.5%) and contained a minimum dry protein content of 91%, was obtained from Arla Foods Ingredients, Viby J, Denmark. Flaxseed oil was purchased from Elixi Oil Oy (Somero, Finland), with the following fatty acid content: 4% 16:0, 3% 18:0, 12% 18:1, 15% 18:2, and 66% 18:3. Laccase was produced by *T. hirsuta* and purified by anion exchange chromatography and hydrophobic interaction chromatography [Frasconi et al. 2010]. Laccase activity towards 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was determined to be 1798nkat/mg of protein. Microbial Tgase Activa MP was purchased from Anjinomoto (Japan) and was further purified at VTT [Lantto et al. 2005]. The activity of the enzyme was determined by colorimetric hydroxymate method as described by Lantto et al. [Lantto et al. 2007]. For the succinylation and the EDA modification of sodium caseinate and whey protein(I, II), succinic anhydride (purity ≥ 97.0%) and ethylene diamine dihydrocloride (purity ≥ 99.0%) were purchased from Sigma. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (purity ≥ 98.0%) was purchased from Pierce. For the vanillic acid modification of whey protein (III), Vanillic acid (purity ≥ 97.0%) was purchased from Sigma-Aldrich. Sulfo-NHS (purity ≥ 98.5%) was purchased from Pierce.

2.2 Modifications on milk proteins

CN was modified by both succinic acid and ethylene diamine respectively (I) and WPI was only modified by ethylene diamine (II). These modifications were performed before the formation of emulsions. The vanillic acid modification of WPI was performed before emulsification and after this the emulsion made of the vanillic acid modified WPI was cross-linked by laccase (III). CN was cross-linked by Tgase before emulsification (IV).

2.3 Characterization of the modified proteins

CN and WPI after succinylation or EDA modification were characterized using sodium docecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 2,4,6-trinitro-benzene sulfonic acid (TNBSA) assay and MALDI-TOF mass spectrometry (MS) (I, II). The extent of the vanillic acid modification of WPI was studied using MALDI-TOF MS (III). The laccase-catalyzed cross-linking was studied using SDS-PAGE, UV/Vis spectrophotometry and oxygen consumption measurement (III). The Tgase-catalyzed cross-linking was studied using SDS-PAGE electrophoresis and ammonia production measurements (IV).

2.4 Preparation of O/W emulsions

The modified and unmodified proteins were solubilized in Milli-Q water at a concentration of 0.3% or 1%. 90% w/w protein solutions were homogenized with 10% w/w flaxseed oil in two steps. A pre-emulsion was prepared using a high speed homogenizer (Heidolph Diax 900, Germany) under constant condition: 2 times 2 min at 26000 rpm. The main emulsification was performed using a pressure homogenizer (Microfluidics M-110Y, USA) at 0 ºC and 40 psig (500 bar) for 10 minutes.

2.5 Analysis of emulsifying properties of the modified proteins and emulsion stability

The emulsifying properties of the modified and unmodified milk proteins and the stability of emulsions made of these proteins were analysed using various techniques. The analytical methods used in this work are summarized in Table 4, according to the purposes and the type of modification.

Table 4. Techniques used to study the emulsifying properties of proteins and emulsion stability.

Method	Aim	Used in publication
Laser diffraction	Determination of particle size distribution, mean droplet diameter and specific surface area	I, II, III, IV
Multiple light scattering	Evaluation of pH & storage stability of emulsions and determination of creaming velocity	II
Zeta-potential measurement	Determination of the surface charge of emulsion droplets	II
Surface protein displacement	Evaluation of the strength of surface protein layer against surfactants	II
Confocal microscopy	Visualization of flocculation and coalescence of emulsion droplets	III
Oxygen consumption measurement	Evaluation of the formation of hydroperoxides in emulsions	IV
Peroxide value measurement	Determination of the accumulation of hydroperoxides, primary oxidation products	IV

Gas chromatography	Formation of volatile secondary oxidation products in emulsions	IV

3. Results

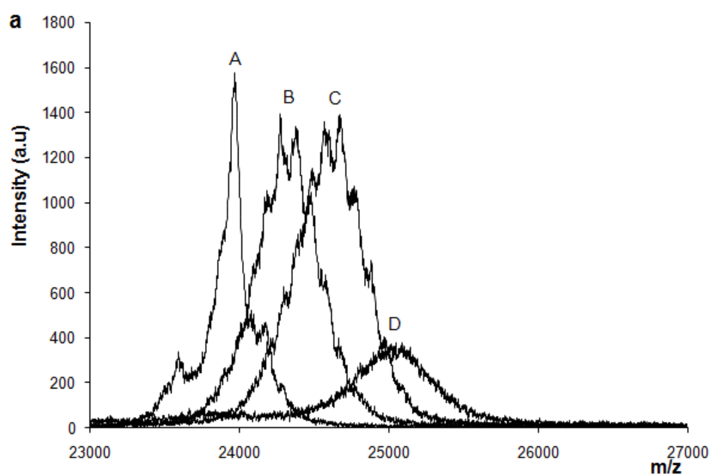
3.1 Succinylation and ethylene diamine modification of milk proteins and their effect on pH stability of emulsions (Publications I&II)

A lot of food emulsions are made at weak acidic pH values (～4.5) where the functionality of milk proteins as an emulsifier is compromised due to the loss of surface charge. Succinylation and EDA modification reactions were carried out in order to shift the pI of milk proteins towards both the acidic and alkaline pH region and thus to allow the interfacial proteins provide enough electrostatic repulsion between emulsion droplets. CN was first modified by both succinic acid and EDA (I). The EDA modification was found to effectively increase the net charge of CN at its pI. However, the modified CN was inevitably cross-linked during the EDA modification which compromised its emulsifying activity. Therefore WPI was modified by EDA to produce a monomeric protein emulsifier with an increased pI (II). The extent of the different modifications was studied by MALDI-TOF MS and SDS-PAGE. After this the emulsifying properties of the unmodified and modified proteins were compared.

3.1.1 Extent of succinylation and EDA modification

The molecular weight (M_w) of the milk proteins was determined by MALDI-TOF MS before and after the reactions to assure the occurrence and measure the extent of the modification (**Figure 4**). The number of the modified residues was estimated by dividing the difference between the average molecular mass of modified and unmodified proteins by the mass increase of each modified residue on the proteins (M_w of the chemical modifier minus 18 Da by the formation of H_2O).The average number of succinylated lysine groups in CN modified with 0.03, 0.06, 0.2g of succinic anhydride per gram of protein was 2.5, 5.7, and 11.5 respectively (**Figure 4a**). Due to poor solubility of unmodified CN at the optimum pH (pH 4.7) for the EDA modification, 0.06g/g succinylated CN was used as the starting material. The average number of carboxyl groups which reacted with EDA was calculated to be 17.2 (**Figure 4b**). For WPI, the EDA modification could be directly carried out using the unmodified protein, since WPI maintained good solubility at pH 4.7. After the modification, there were on average 7.8 and 15.6 carboxyl groups in α-lactalbumin and β-lactoglobulin modified by EDA respectively (**Figure 4c**).

CN is a complex mixture of different casein variants (α, β, κ casein), therefore the spectra measured using MALDI-TOF MS actually represent the overall population of the unmodified or modified variants rather than a mixture of equally modified proteins. The extent of succinylation could be controlled and CN was gradually succinylated using an increasing amount of succinic acid as the modifier. As indicated by the reduction of the peak size going from A to D in Figure 4a, more and more laser power was required in a positive ion mode in MALDI-TOF MS to generate mass spectra from succinylated caseinates with an increasing extent of modification. No reliable spectrum from the modified proteins could be measured after the succinic anhydride/protein ratio exceeded 0.2 g succinic anhydride per gram of protein (**Figure 4a**). On the contrary, incorporating positively charged groups onto the protein seemed to be an efficient way to enhance the signal of proteins in MALDI-TOF MS. After the EDA modification, for both CN and WPI, sound spectra were generated with lower laser power requirement (**Figure 4b&c**).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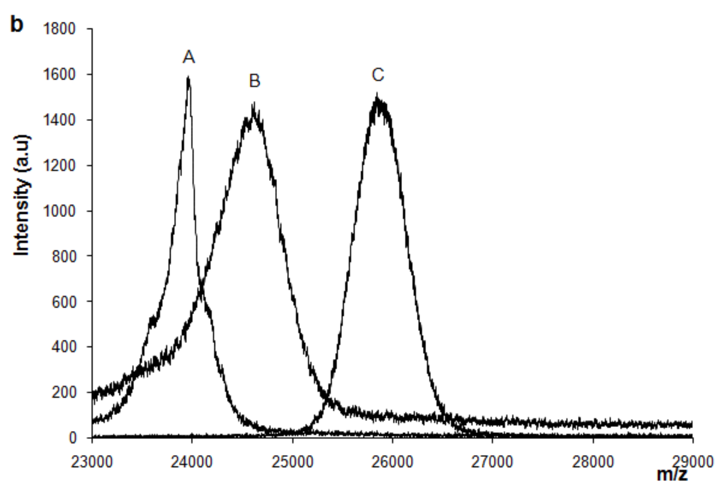
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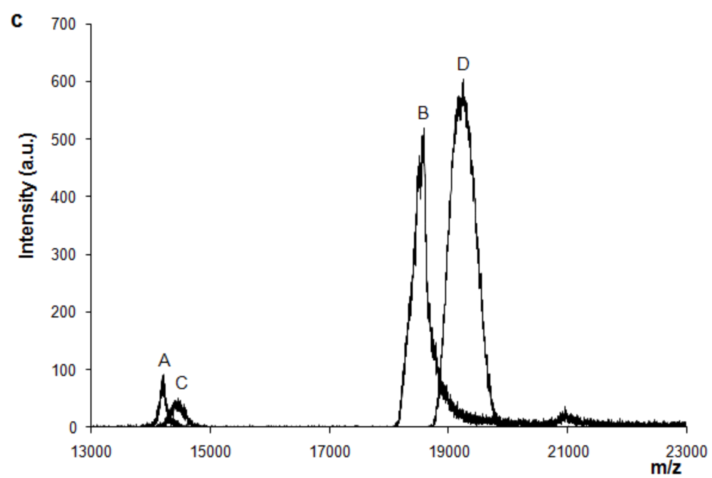
c
Intensity (a.u.)
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Figure 4. MALDI-TOF mass spectra of modified milk proteins. (**a**) Succinylation of CN. Spectrum A, CN control with molecular weight 23966 Da; spectrum B, succinylated CN by 0.03 g succinic anhydride per gram of CN with molecular weight 24211Da; spectrum C, succinylated CN by 0.06 g succinic anhydride per gram of CN with molecular weight 24529 Da; spectrum D, succinylated CN by 0.2 g succinic anhydride per gram of CN with molecular weight 25089 Da. (**b**) EDA modification of CN. Spectrum A, CN control with molecular weight 24148Da; spectrum B, succinylated CN by 0.06 g succinic anhydride per gram of CN with molecular weight 24584Da; spectrum C, EDA modified succinylated CN using 0.06 g succinic anhydride per gram of CN with molecular weight 25853Da. (**c**) EDA modificaion of WPI. Spectrum A, α-lactalbumin of the unmodified WPI with molecular weight 14229 Da; spectrum B, β-lactoglobulin of the unmodified WPI with molecular weight 18579 Da; spectrum C, α-lactalbumin of the EDA modified WPI with molecular weight 14527 Da; spectrum D, β-lactoglobulin of the EDA modified WPI with molecular weight 19250 Da.

The EDA modification showed a side reaction in which the proteins were cross-linked to a different extent. The cross-linked products could not be detected in MALTI-TOF MS but were observed in the SDS-PAGE gel (**Figure 5**). CN was extensively cross-linked which was most probably due to its open structure. A large fraction of the EDA modified CN was polymerized into large molecules which could not migrate in the gel (**Figure 5, lane 5**). As comparison, WPI, which has a compact globular structure, was cross-linked to a lesser extent. Most of the protein after the reaction existed as EDA modified monomers and very little was cross-linked into dimers or oligomers (**Figure 5, lane 7**).

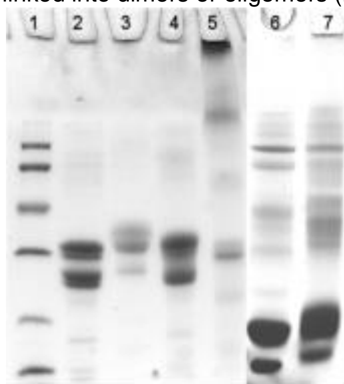
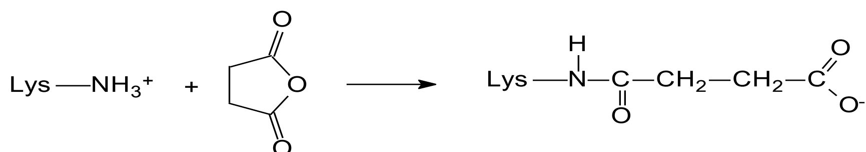


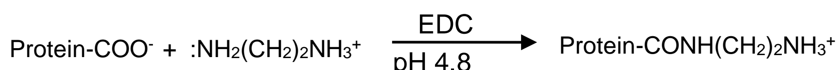
Figure 5. Succinylation and EDA modification of CN and WPI.Lane 1, low molecular weight marker proteins of 97, 67, 45, 30 and 20 kDa; Lane 2, unmodified CN control; Lane 3, succinylated CN by 1 g of succinic anhydride per gram of CN; Lane 4, succinylated CN by 0.06 g of succinic anhydride per gram of CN; Lane 5, EDA modified CN; Lane 6, unmodified WPI control; Lane 7, EDA modified WPI.

3.1.2 Effect of succinylation and EDA modification on the pI of proteins

In the succinylation reaction, a positively charged ε-amino group of a lysine residue reacts with the negatively charged succinic acid. The modified proteins contain negatively charged side groups, resulting in a decreased pI (**Scheme 1**). The EDA modification converts a negatively charged carboxyl groups into a positively charged amino group and the pI of the protein shifts to a higher pH value (**Scheme 2**).



Scheme 1. Succinylation of proteins.



Scheme 2. EDA modification of proteins.

The zeta potential of these unmodified and the modified CN and WPI variants was determined using a Zetasizer and plotted as a function of pH (**Figure 6**). When the measurement is carried out using a very low ionic strength, the pH value at which the zeta potential was zero corresponds with the pI of the protein [Salis et al. 2011]. As a result of the modification reactions, the succinylation to full extent altered the pI of CN from 4.2 to 2.7, and the EDA modification shifted the pI of CN and WPI from 4.2 to 9.4 and from 4.9 to 9.5 respectively. Within the pH range close to the pI (pI±0.5), the zeta-potential of the proteins varied less than 10 mV, indicating a weak electrostatic repulsion between the protein molecules [Sherman 1970]. On the contrary, at pH values far from the pI, the intermolecular repulsion was strong due to the extensive charge of the proteins.

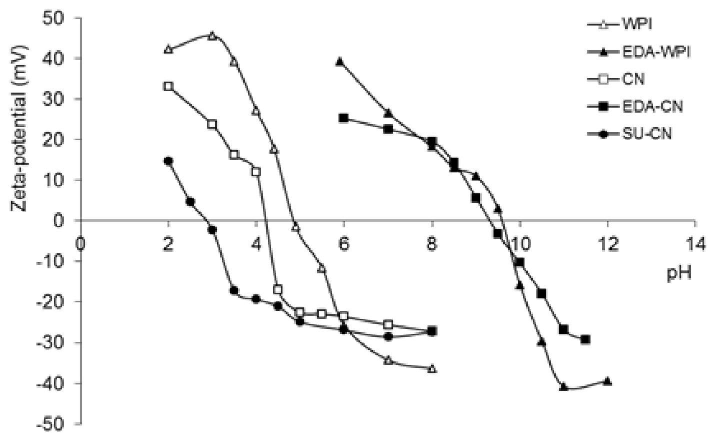


Figure 6. Zeta-potential of unmodified and modified CN and WPI. Zeta-potential of unmodified CN (□), succinylated CN (SU-CN) (●), EDA modified CN (EDA-CN) (■), unmodified WPI (△) and EDA modified WPI (EDA-WPI) (▲) was measured and plotted as a function of pH.

3.1.3 Emulsifying properties of the modified proteins

The EA of the modified CN and WPI was evaluated by comparing the mean particle size of freshly made emulsions, and the ES of emulsions was evaluated by following the increase of particle size after three-day storage (**Table 5**). An efficient emulsifier should be able to create stable emulsions with small droplet size (less than 1µm) which does not significantly increase over time [Stauffer 1999]. Both CN and WPI have a pI close to pH 5 and therefore the unmodified proteins failed to make stable emulsions at this pH value due to the loss of net surface charge. The droplet diameter of the fresh emulsions was already very large from the start (over 10 µm) and with a high rate of aggregation and creaming, the droplets eventually formed a thick cream layer at the top of the emulsions and clarification was observed at the bottom after storage. At pH 3 and 7, the unmodified proteins carried enough charge to stabilize the O/W interface. Both proteins showed a better EA and ES at pH 7 than at pH 3 as can be seen from the smaller droplet size of the fresh and stored emulsions.

Succinylation of CN improved the EA and the stability of CN stabilized emulsions at pH 5 and 7, especially at pH 5 where the droplet size significantly decreased compared to the emulsions made of the unmodified CN. However, in agreement with the zeta-potential results, the succinylated CN lost its net charge at pH 3 and a severe flocculation of oil droplets followed by phase separation occurred right after the emulsification process.

For both CN and WPI, the EDA modification shifted the pH stability profile of the emulsions towards the alkaline pH region, resulting in improved ES at pH 3 and 5. However, the EA of the EDA modified CN was compromised due to the formation of large polymer proteins caused by the cross-linking side reaction, which could retard the adsorption of proteins on the interface and leads to a less efficient surface coverage. Therefore, using limited amount of protein (0.3%), only a limited surface area could be created and stabilized. As shown in the SDS-PAGE gel result in **Figure 5**, the EDA modified WPI was mostly produced as monomers. During the emulsification process, these protein monomers could readily adsorb onto the O/W interface at a similar rate to the unmodified WPI, stabilizing the new created interface with extensive electrostatic charge all through the acidic and neutral pHs. As a result, both EA and ES were improved by the EDA modification on WPI.

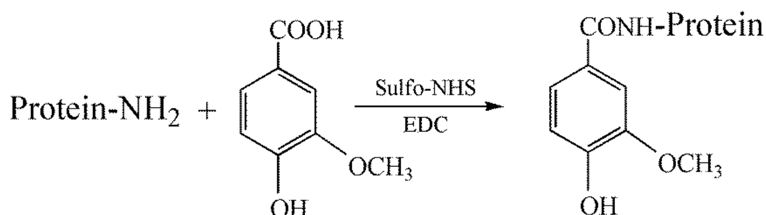
Table 5. Mean droplet diameter of emulsions made of the unmodified and modified proteins.

	CN (μm)		SU-CN (μm)		EDA-CN (μm)		WPI (μm)		EDA-WPI (μm)	
	0d	3d	0d	3d	0d	3d	0d	3d	0d	3d
pH 3	0.68	1.4	ND	ND	0.46	0.80	0.65	0.77	0.39	0.43
pH 5	10.6	34.2	1.4	3.2	1.4	2.9	13.1	ND	0.48	0.50
pH 7	0.39	0.42	0.31	0.33	1.2	10.0	0.33	0.43	0.78	0.93

ND: Not determined, phase separation took place right after the pre-emulsification process. Unmodified CN (CN), succinylated CN (SU-CN), EDA modified CN (EDA-CN), unmodified WPI (WPI) and EDA modified WPI (EDA-WPI) at a protein concentration of 0.3% was used.

3.2 Cross-linking of milk proteins by laccase and Tgase (Publications III&IV)

Besides the chemical modifications described in section 3.1, the milk proteins were enzymatically modified by laccase and Tgase. WPI, which has low reactivity towards laccase, was first modified by vanillic acid (**Scheme 3**) to study whether this could increase the efficiency of the laccase catalyzed cross-linking. The cross-linking of the vanillic acid modified WPI was compared with the laccase catalyzed cross-linking of unmodified WPI, or with the cross-linking by adding laccase together with free phenolic compounds as mediators (**Figure 7**). On the other hand, CN was directly cross-linked by Tgase (**Figure 9**). The extent of these cross-linking reactions was studied using SDS-PAGE gel.



Scheme 3. Vanillic acid modification of proteins. The reaction is mediated by N-hydroxysulfosuccinimide sodium (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).

3.2.1 Laccase catalyzed cross-linking of WPI and the vanillic acid modified WPI

The reactivity of WPI towards laccase was very low. At a high laccase dosage of 2.5 nkat/mg, only a slight increase of the band intensity of dimers was observed after 24 h incubation, indicating a very small extent of cross-linking of the proteins (**Figure 7a, lane 7**). The low reactivity could be attributed to the fact that the laccase reactive tyrosine residues of the unmodified WPI might be buried inside its compact globular structure and not accessible for the enzyme. Addition of free vanillic acid improved the cross-linking to a limited extent (**Panel b**). The intensity of the dimer band was slightly higher compared to the WPI sample without this mediator and a little amount of protein polymer was detected in the upper part of the lane (**Figure 7b, lane 7**). The vanillic acid modification was shown by MALDI-TOF MS to incorporate 2.9 and 2.1 methoxylphenol side groups into one molecule of α-lactalbumin and β-lactoglobulin according to the same calculation method in section 3.1.1 (**Figure 8**). This modification occurred most likely on the surface of the proteins making the incorporated groups more accessible for laccase. As a result of this, the laccase catalyzed cross-linking of the vanillic acid modified WPI was significantly more effective than that of the unmodified WPI or the combination of unmodified WPI and free vanillic acid (**Figure 7c**). Using the same dosage of laccase, the cross-linking reaction after 2 h incubation was much more extensive than that of the other samples incubated for 24 h shown in Panel a&b (**Figure 7c, lane 3**), and the molecular weight of the cross-linked proteins ranged from 29 kDa to over 206 kDa.

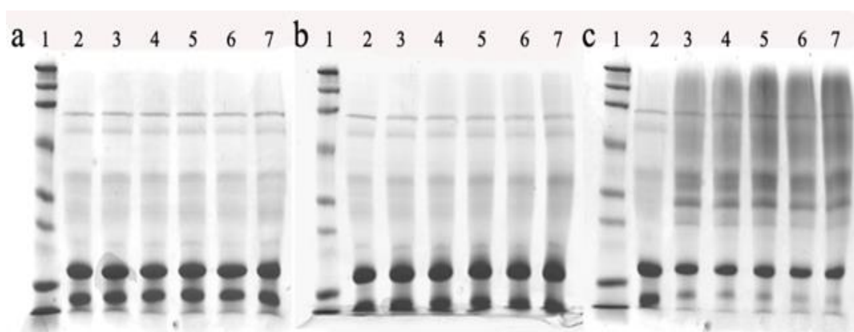


Figure 7. SDS-PAGE of the unmodified and modified WPI after laccase reaction. The cross-linking was performed at a laccase dosage of 2.5 nkat/mg. (**a**) cross-linking of the unmodified WPI. (**b**) cross-linking of the unmodified WPI with 2 mM free vanillic acid. (**c**) cross-linking of the vanillic acid modified WPI. Lane 1, prestained SDS-PAGE standard proteins of 206.3, 118.1, 97.3, 54.8, 37.7, 29.4, 17.5, and 6.7 kDa (BIO-RAD); Lane 2–7, cross-linked samples at 0, 2, 4, 8, 12 and 24 h.

Compared to addition of free phenolic compounds as a mediator between laccase and WPI, the vanillic acid modification directly on WPI enhanced the laccase catalyzed cross-linking more efficiently. Furthermore, using free vanillic acid as a mediator and/or bridging component had the disadvantage that the self-polymerization of these compounds caused color formation which could be undesirable for some applications. Cross-linking of vanillic acid modified WPI was based on a direct radical formation in the protein and therefore the color formation was avoided.

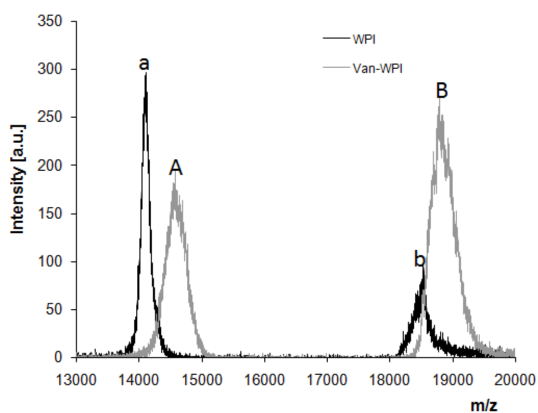


Figure 8. MALDI-TOF mass spectra of the modified WPI. Spectrum a, unmodified α-lactalbumin with molecular mass 14118 Da; Spectrum b, unmodified β-lactoglobulin with molecular mass 18481 Da; Spectrum A, vanillic acid modified α-lactalbumin with molecular mass 14557 Da; Spectrum B, vanillic acid modified β-lactoglobulin with molecular mass 18798 Da.

3.2.2 Tgase catalyzed cross-linking of CN

CN, due to its open flexible structure, is more susceptible to Tgase and laccase than WPI. In this study, CN was cross-linked overnight by Tgase to different extents, depending on the enzyme dosage (**Figure 9**). The extent of the cross-linking reaction increased with an increasing dosage of Tgase. At a Tgase dosage of 50 nkat/g, a slight increase of the intensity of the protein oligomer bands was observed with M_w from 97 to 206 kDa, but most of the CN was still uncross-linked (**Figure 9, lane 3**). At a dosage of 500 nkat/g Tgase, most of the CN had been cross-linked into high molecular weight oligomers (~206 kDa) and only a small fraction of the CN stayed uncross-linked. Extensively cross-linked products with very large molecular mass could not migrate in the SDS gel and were observed on the top of the lane (**Figure 9, lane 5**).

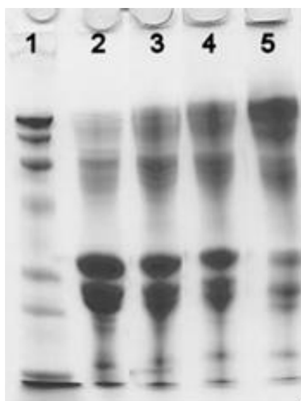


Figure 9. SDS-PAGE of the cross-linked sodium caseinate by Tgase. Sodium caseinate was treated with Tgase at an enzyme dosage of 50, 100 and 500 nkat/g at RT overnight. Lane 1, prestained SDS-PAGE standard proteins of 206.3, 118.1, 97.3, 54.8, 37.7, 29.4, 17.5 and 6.7 kDa (BIO-RAD); lane 2, non-cross-linked sodium caseinate control; lane 3, cross-linked sodium caseinate with 50 nkat/g Tgase; lane 4, cross-linked sodium caseinate with 100 nkat/g Tgase; lane 5, cross-linked sodium caseinate with 500 nkat/g Tgase.

3.3 Effect of cross-linking on emulsion stability (Publications III&IV)

As protein emulsifiers, CN and WPI were cross-linked by Tgase and laccase respectively. The cross-linking of Van-WPI by laccase was applied after the formation of emulsion, whereby the physical stability of the emulsion was enhanced. CN was cross-linked by transglutaminase prior to the emulsification process, resulting in an improved oxidative stability.

3.3.1 Effect of cross-linking on the physical stability of emulsions

Droplet behaviour in the laccase cross-linked and the non-cross-linked emulsions was visualized using confocal laser scanning microscope (CLSM) to compare the physical stability of these two emulsions (**Figure 10**). At 0 h, the distribution of the oil droplets in both cross-linked and non-cross-linked emulsions was homogeneous and the diameter of most of the oil droplets was less than 1 μm (**Figure 10a & d**). During 72 h storage, coalescence was observed in those emulsions in which the interfacial proteins were not cross-linked (**Figure 10b, c and e**), whilst the laccase treated Van-WPI emulsion showed less droplet aggregation and no larger particles were visible (**Figure 10f**). This result was in agreement with the result of average particle size measured by the laser diffraction method and the visual observation that a thick oil layer was formed on top of the non-cross-linked emulsions while no creaming was observed in the cross-linked emulsion during 1 week storage (**Figure 11**). The post-emulsification cross-linking by laccase was assumed to occur between the adsorbed protein and free protein molecules in the water phase, resulting in a denser and more viscoelastic interfacial protein layer that provided stronger physical barrier against droplet coalescence. On the other hand, the cross-linking between the proteins in the continuous phase might lead to the formation of a further extended protein structure which increased the viscosity of the continuous phase to some extent and limit the rate of aggregation and creaming behaviour of the oil droplets.

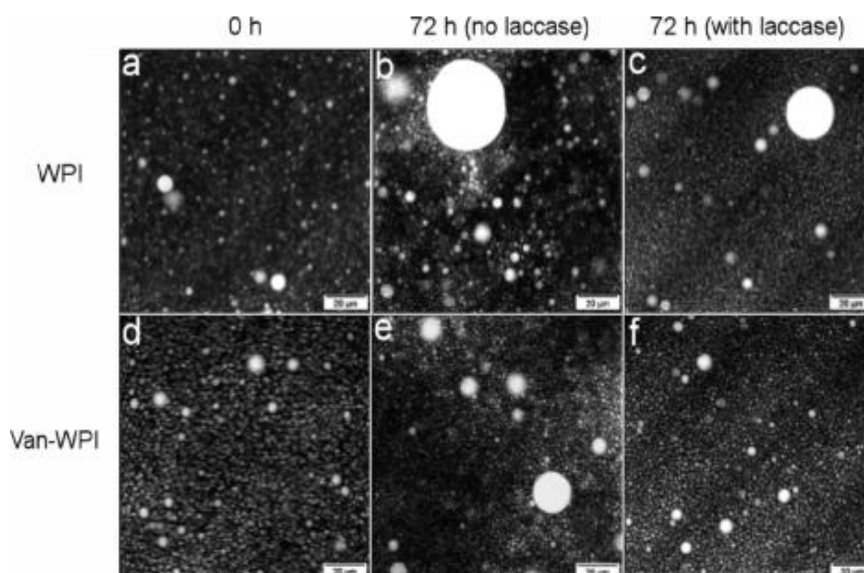


Figure 10. Confocal laser scanning microscope images of emulsions made of the unmodified and modified WPI with or without laccase treatment. Size bar = 20 μm. Nile Red was used to stain the oil phase = bright regions. (**a–c**) Different emulsions made from WPI; (**d-f**) different emulsions made from Van-WPI.

Figure 11. Visual observation of aged emulsions. The emulsion samples were stored at RT for 1 week. Clarification and creaming were observed respectively from the bottom and the top of tubes.

CN was cross-linked by Tgase at a dosage of 500 nkat/g (TGCN) prior to the emulsification process. After the emulsions made of the non-cross-linked and cross-linked CN were prepared under the same emulsification conditions and the droplet size of these two emulsions was followed for 30 days (**Figure 12**). At high protein concentration (1%), the pre-emulsification cross-linking did not affect the emulsifying activity of the modified proteins since the mean droplet diameter of the

fresh TGCN emulsion was the same as that of the CN emulsion (0.16 µm). The physical stability of emulsions was slightly improved by the Tgase treatment. After 30 days storage, the mean droplet diameter increased to 0.29 µm for the TGCN emulsion and 0.38 µm for the CN emulsion. This improvement of the physical stability of emulsions could be attributed to a denser interfacial layer formed by the cross-linked proteins.

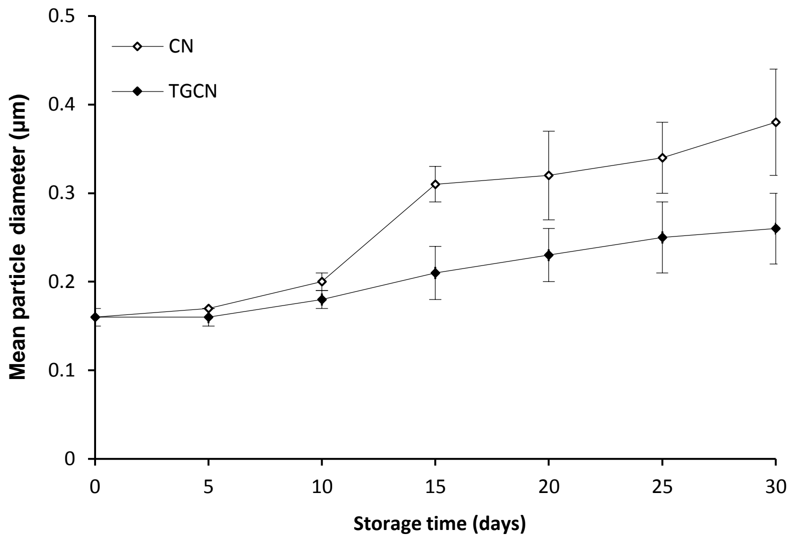


Figure 12. Physical stability of emulsions over 30 days storage. The emulsions made of non-cross-linked sodium caseinate (CN) (◇) and cross-linked sodium caseinate (TGCN) (◆) was stored at room temperature in dark for 30 days. Samples were taken every 5 days and the volume-surface mean particle diameter (d32) was calculated from volume distribution of three batches of emulsions. Two measurements of particle size distribution were conducted from each batch.

3.3.2 Effect of cross-linking on the oxidative stability of emulsions

Based on the measurement of luminescence quenching by oxygen in the aqueous phase, the oxygen consumption in the emulsions made of the non-cross-linked CN and the cross-linked CN was followed as an indication for their oxidative stability (**Figure 13**). In an O/W emulsion system where oxygen is consumed in the oil phase for lipid oxidation, the oxygen in the aqueous phase will diffuse into the oil phase until an equilibrium state is reached. Therefore, the decrease of the concentration of oxygen in the aqueous phase is assumed to be due to the formation of fatty acid hydroperoxides, which is the oxygen consuming step of the lipid oxidation reaction. The emulsions were stored under conditions where a large air space and constant stirring allowed quick and sufficient oxygen supply to the aqueous phase so that availability of oxygen did not become limiting for the reaction and the oxygen concentration in the emulsion samples for the following oxygen consumption measurement started from the same saturated level. The CN emulsions went through an induction phase within the first 10 days of storage during which the rate of oxygen consumption slightly increased from 0.34 to 1.01mmol/L·day (**Figure 13**). After this time point, the oxidation rate rapidly increased and oxygen consumption rate reached 68.7 mmol/L·day for the CN emulsion after 30 days of storage. The lipid oxidation in the TGCN emulsion was significantly inhibited and the oxygen consumption rate was at a much lower level within these 30 days.

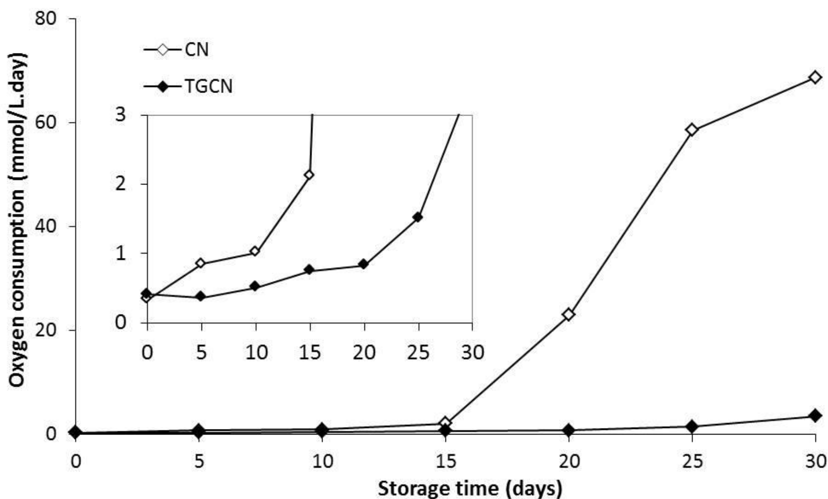


Figure 13.Oxygen consumption measurement. The oxygen consumption in emulsions stored for 0, 5, 10, 15, 20, 25 and 30 days were monitored using a single-channel oxygen meter. The consumed oxygen was plotted against time and the rate of oxygen consumption was calculated as the slope of the linear part of each plot. The unit was presented in millimole of oxygen consumed in one liter of emulsions per day.

Other methods such as peroxide value measurement (**Figure 14**) and headspace gas chromatography analysis of the volatile oxidation products (**Figure 15**) were applied to confirm the inhibiting effect of cross-linking on the lipid oxidation. The results were in good agreement with the oxygen consumption data. The concentration of fatty acid hydroperoxides stayed at a relatively low level during the first 15 days storage for both CN emulsion and TGCN emulsion, whereas the formation of peroxides in the CN emulsion accelerated after the induction phase and reached a considerably higher level (386 meq/kg oil) after 26 days compared to the TGCN emulsion (50 meq/kg oil).

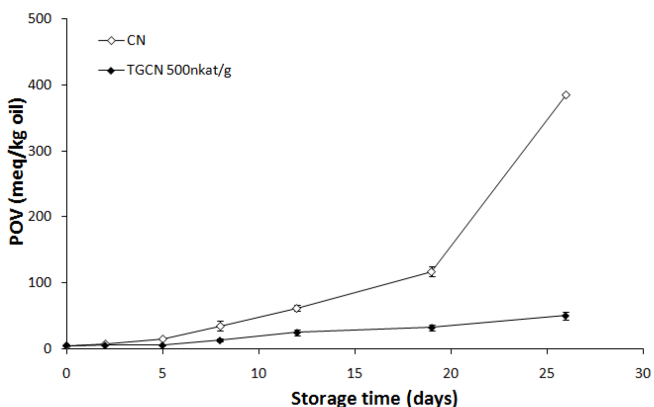
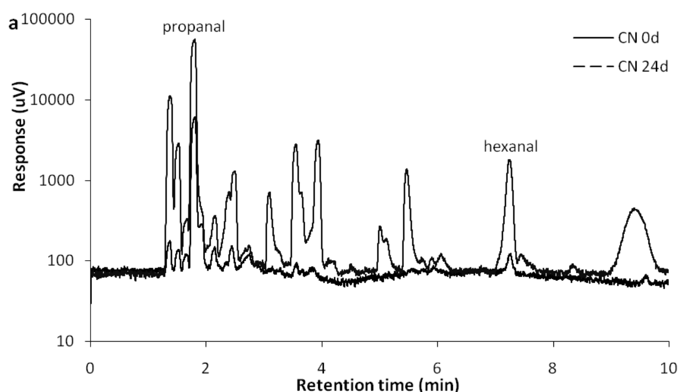


Figure 14. Oxidation measured as peroxide values of flaxseed oil emulsions as stored without light exposure at 20°C with mixing and large airspace to ensure supply of oxygen to continuous phase.

A complex mixture of secondary oxidation products was detected since the flaxseed oil contains different types of unsaturated fatty acids. The peaks corresponding to propanal and hexanal were identified in the chromatograms and the peak area was calculated as an indication of the extent of lipid oxidation. For the CN emulsion after 24 days storage, the peak area of propanal and hexanal increased from 2.8×10^4 to 2.7×10^5 and from nearly 0 to 1.1×10^4 respectively, with a number of unidentified peaks of other oxidation products (**Figure 15a**). For the TGCN emulsion the oxidation was effectively slowed down as indicated by a significantly less growth of these peaks. After 24 days storage, the peak area of propanal and hexanal increased from 1.1×10^4 to 1.6×10^4 and from 0 to 1300 respectively (**Figure 15b**).



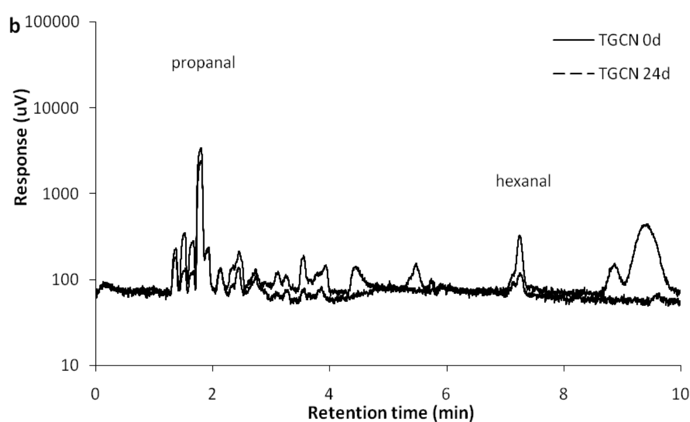


Figure 15. Headspace analysis. The secondary products of lipid oxidation were followed by head space gas chromatography. The whole chromatograms of fresh emulsions prepared with non-cross-linked (panel a) and cross-linked sodium caseinate (panel b) and the emulsions after 24 days storage were presented by plotting the detector response against retention time.

4. Discussion

Proteins stabilize an emulsion by forming a charged and viscoelastic layer at the O/W interface. This interfacial layer offers electrostatic repulsion and steric hindrance to protect the droplets against flocculation when they come close to each other, and meanwhile the viscoelasticity of the layer prevent the droplets from coalescence during droplet collision. The interfacial proteins, as a physical barrier against the penetration of pro-oxidants or oxygen, could help to retard the lipid oxidation. In this work, succinylation and EDA modification of milk proteins was carried out to increase the electrostatic repulsion of the interfacial proteins at pH values close to the pI of the unmodified proteins, and as a result the pH stability of emulsions was improved. Enzymatic cross-linking before or after emulsification was proven to enhance the mechanical properties of the interfacial protein layer and led to an improved physical stability or oxidative stability of the emulsions.

4.1 Chemical and enzymatic modifications on milk proteins

4.1.1 Modification of milk proteins through succinylation and EDA modification

Succinylated milk proteins could be produced under relatively simple conditions. This modification did not require other catalysts or reagents and the proteins were modified instantaneously without formation of byproducts. As shown by MALDI-TOF MS, the extent of succinylation could be subtly controlled by adding different amounts of succinic anhydride and with an excess of succinic anhydride almost all the amino groups could be converted into carboxyl groups. The increasing extent of succinylation with the concentration of succinic anhydride measured using TNBSA method [Hall et al. 1977] (data not shown) was in agreement with a previous study in which 86% succinylation was carried out for β-casein at 0.2:1 weight ratio of succinic anhydride to the protein [Hoagland 1968]. For higher extent of succinylation extensive amount of succinic anhydride was required. This result correlates well with literature studies [Mühlrad et al. 1968, Groninger 1973]. In food industry, succinylated monoglycerides are produced via the reaction between distilled monoglycerides with succinic anhydride, improving the emulsifying performance of monoglycerides at acidic pH values. The improved emulsifying properties of succinylated CN suggest that the succinylation method could expand the use of protein emulsifiers in the same way.

For the modification with EDA, the carboxyl groups of a protein need to be first activated by EDC, forming an *O*-acylisourea intermediate which could then react with the primary amine of an EDA molecule. In this work, the poor solubility of CN at the optimal pH for the EDA modification was overcome by applying a partial succinylation to the protein prior to the target reaction. Partially succinylated CN had an improved solubility at pH 4.8 and more carboxyl groups that were converted into amino groups. In comparison with CN, WPI aggregated to a lesser extent resulting in no precipitation at pH 4.8. Therefore, WPI could be directly used for the EDA modification. For both CN and WPI, excess EDA was required to make the reaction proceed and it was estimated that nearly half of the carboxyl groups could be modified using this method. The products of the EDA modification are largely dependent on the structural nature of the target proteins. During the EDA modification, intermolecular polymerization of CN was formed due to a EDC induced cross-linking side reaction. The high surface hydrophobicity would increase the possibility of a close approach between two CN molecules and then their open structure would facilitate the formation of amide bonds between the EDC activated carboxyl groups and amino groups on other CN molecule. For WPI which has a less hydrophobic surface and a compact globular structure, the intermolecular cross-linking of two WPI molecules seems to be effectively prevented due to the steric hindrance. Therefore, the EDC activated carboxyl groups might be only accessible for small EDA molecules and eventually an EDA-WPI was formed as a modified protein monomer.

Other modifications to increase the pI of proteins are esterification which blocks the negatively charged carboxyl groups and Maillard reaction between

protein and cationic saccharides. Mattarella et al. [1983] studied the effect of esterification on the pI of bovein β-lactoglobulin. The protein's pI was shifted from 5.2 for the unmodified β-lactoglobulin to 8.7 and 9.5 respectively for ethyl-esterified and methyl-esterified β-lactoglobulin derivatives. Hattori et al. [2000] found that the pI of β-lactoglobulin could be shifted to 5.0, 6.0 and 7.5 after being modified by cationic glucosamine, chitopentaose and chitosan respectively. This Maillard reaction produces protein-cationic saccharides conjugates at expense of losing intrinsic positively charged amino groups of the protein. This might explain why the Maillard reaction is less effective in shifting the pI of proteins into alkaline region compared to esterification and EDA modification.

4.1.2 Cross-linking of milk proteins by laccase and transglutaminase

In this thesis work, CN and WPI were cross-linked by Tgase and laccase respectively. The reactivity of milk protein substrate towards the laccase and Tgase was dependent on the accessibility of the reactive residues in the proteins to the enzyme. In the case of laccase which reacts with aromatic substrates, tyrosine alone or a short peptide containing exposed tyrosine residues can be directly cross-linked by laccase [Mattinen et al. 2005]. In a globular protein such as WPI, the partially hydrophobic tyrosines are typically buried in the hydrophobic core of the protein. The structure of β-lactoglobulin suggested that only one out of 4 total tyrosine residues was fully exposed to the solvent at pH 6.5, and this exposed tyrosine may not be accessible for the active site of laccase [Brownlow et al. 1997]. This poor accessibility of tyrosines in WPI could explain the very limited extent of cross-linking of the unmodified WPI by laccase in this work (III). A common approach to enhance the laccase-catalyzed cross-linking of protein substrates is to add free phenolic compounds as mediators [Bourbonnais & Paice 1990, Selinheimo et al. 2008b]. These small phenolic compounds could be first oxidized by laccase and then transfer electrons as they diffuse and reach reactive residues such as tyrosine and cysteine inside the protein structure. However, this approach facilitates only the radical formation stage of the cross-linking. The non-enzymatic reaction which eventually leads to the formation of intermolecular cross-links was still limited due to the poor accessibility of the formed radicals to each other. Therefore, the addition of free phenolic mediators improved the cross-linking of WPI only to a small extent, and as a side effect, color change was observed due to the chemical changes of phenolic compounds. The color change could be attributed to the formation of quinone components during the laccase catalyzed oxidation of phenolic compounds and this reaction was utilized to synthesize colorant [Mustafa et al. 2005].

In this work, the reactivity of WPI towards the laccase enzyme was effectively improved by incorporating artificial methoxylphenol groups onto the protein structure via vanillic acid modification. The methoxyphenol groups are assumed to be incorporated onto the protein surface and thus fairly accessible for laccase. These new reactive sites could be directly oxidized into radicals and then form intermolecular cross-links with other oxidized protein molecules. Since the cross-linking of vanillic acid modified WPI was based on a protein-bound radical polymerization without addition of any free phenolic compounds color formation could be avoided. The vanillic acid modification could broaden the use of the laccase catalyzed cross-linking of proteins where color formation is undesired. In addition, the cross-linking of vanillic acid modified protein implies an opportunity to create biopolymers through modifications of 'protein-lignin' hybrids. Kim and Cavaco-Paulo [2012] synthesized a functional biopolymer via the oxidative conjugation of protein-flavonoid by laccase. The resultant protein-flavonoid

conjugates exhibited new color generation and antioxidant activity in flax fibre applications.

Compared to laccase, Tgase is more commonly used as a cross-linking enzyme for protein substrates which contain more accessible and reactive amino acids towards the enzyme. Again, the reactivity of CN towards Tgase was found to be much higher than that of WPI for the same reasons as discussed above. In this study, native CN was proven to be extensively cross-linked into polymers by transglutaminase (IV). Whereas, under the same enzyme dosage and reaction conditions the cross-linking of WPI was very limited. Similar results were obtained by Færgemand et al. [1997]. To increase the extent of cross-linking of WPI, several methods have been reported to unfold the globular structure including treatments with dithiothreitol, alkaline pH, heat or high hydrostatic pressure [Jaros et al. 2006]. When WPI was used as an emulsifier, the adsorbed WPI on emulsion droplets were also found to be more susceptible to Tgase since the proteins unfolded at the O/W interface and thus had better accessibility for the enzyme [Færgemand et al. 1998b]. Instead of physically unfolding the protein structure, another approach to improve the reactivity of WPI towards Tgase could be adding additional reactive sites at positions more exposed to Tgase. For this reason, it would be interesting to study the reactivity of the EDA modified WPI towards Tgase since the modification adds artificial amino groups most likely onto the surface of the proteins.

4.2 Effect of droplet surface charge in a protein stabilized emulsion

Using modifications with succinic anhydride or EDA, the sign and magnitude of the surface charge of interfacial proteins could be manipulated at a given pH. For example, succinylated and unmodified CN or WPI stabilized emulsions at neutral or alkaline pH values with sufficient negative charge on the droplet surface, whilst EDA modified protein emulsifiers functions better at acidic and neutral pH values with extensive positive charge. In food industries where emulsions are often produced in weak acidic or neutral pH range, the EDA modified proteins with an increased pI might be more practical than succinylated ones whose pI was lowered. Besides the magnitude, the sign of surface charge could also plays an important role in the emulsion stabilities since it is an important factor influencing the interactions between the droplet surface and the components in the aqueous phase. Ideally, the interfacial proteins should take the same type of charge as the main components in the aqueous phase that undermine the emulsion quality. One example would be transition metals that could accelerate the lipid oxidation by catalyzing the decomposition of lipid hydroperoxides [McClements & Decker 2000]. A positively charged interfacial protein layer, for example the EDA modified CN or WPI at pH 1–8, could repel these metal ions and thus retard the oxidation of oil.

4.3 Effect of cross-linking on the emulsifying properties of protein

In this work cross-linking by EDC and transglutaminase was applied before the emulsification process and the cross-linking by laccase was performed after the formation of emulsions. The different effect of the pre- and post-emulsification cross-linking on the emulsifying activity and emulsion stability is discussed in this section.

4.3.1 The effect of pre-emulsification cross-linking on EA and ES

For sodium caseinate, the pre-emulsification cross-linking by EDC as a side effect of the EDA modification was proven to weaken EA and ES (I) whilst the pre-emulsification cross-linking by transglutaminase showed no apparent impact on EA but an improved ES (IV). This difference could be attributed to the extent of cross-linking and the concentration of proteins used for emulsification. Extensive cross-linking by EDC resulted in a retarded adsorption of CN polymers with high concentration of these polymers covering a unit area of droplet surface compared to the unmodified CN. This effect was obvious when insufficient protein (0.3%) was used to cover the entire newly created oil surface. As a result, the droplets were more prone to coalescence when they collided with an “unprotected” surface and instantaneous coalescence with a consequent phase separation took place during the homogenization (I). Compared to the extensively cross-linked CN polymers by EDC, the CN was cross-linked by Tgase to a lesser extent. As can be seen from the SDS PAGE gel results, the EDC cross-linked CN as a protein polymer did not migrate in the gel but stayed at the top of the well (**Figure 5, lane 5**), whilst the Tgase cross-linked CN as a protein oligomer migrated to the same level as the 206KDa marker (**Figure 9, lane 5**). This moderate extent of protein polymerization might also retard the adsorption of proteins onto the O/W interface but this adverse effect was overcome using an excessive amount of the cross-linked proteins (1%) to saturate the droplet surface. As a result, the Tgase modified CN exhibited a similar EA to the unmodified one with a slightly improved ES which could be attributed to a denser interfacial layer with stronger steric hindrance between oil droplets. (IV). A conclusion might be drawn based on the comparison above that the pre-emulsification cross-linking reaction could improve the stability of emulsions only if excessive proteins are available for emulsification.

4.3.2 The effect of post-emulsification cross-linking on ES

In this work, the vanillic acid modified WPI was cross-linked into oligomers by laccase after the emulsification process, resulting in an improved storage stability of the emulsion. Theoretically, the post-emulsification cross-linking could occur between adsorbed proteins, between adsorbed proteins and non-adsorbed proteins, or between non-adsorbed proteins. Intermolecular cross-linking occurs between adsorbed proteins on adjacent oil droplets would lead to an impaired emulsion stability by bridging flocculation, which was not in agreement with the stability result observed in this study. Therefore, the cross-linking was first assumed to take place between the adsorbed and the non-adsorbed proteins which led to an increase of the thickness of the interfacial layer and between the non-adsorbed proteins which led to an increased viscosity of the aqueous phase (III). These two types of cross-linking could improve the emulsion stability due to stronger steric hinderance against droplet aggregation and slower creaming velocity. However, later results suggested that at low WPI concentration (0.3%), most of the WPI had adsorbed onto the O/W interface and there was only a very small amount of non-adsorbed proteins remaining in the aqueous phase (II).The contribution of non-adsorbed protein involved cross-linking, even if there was, to the improved stability was insignificant. The improved storage stability of the Van-WPI emulsion should be mainly attributed to the intermolecular cross-linking between the adsorbed proteins which increase the cohesiveness and viscoelasticity of the interfacial film. This speculation is in agreement with a previous study where a Tgase catalyzed post-emulsification cross-linking was proven to occur mainly at the O/W interface [Kellerby et al. 2006].

4.3.3 The effect of pre- and post-emulsification cross-linking on the oxidative stability of the emulsions

Compared to the non-modified CN emulsion, the oxidative stability of the emulsion prepared with Tgase cross-linked CN was significantly improved, as indicated by the much slower rate of oxygen consumption and the production of less secondary products from lipid oxidation(IV).The particle size of CN emulsion and TGCN emulsion was similar for the first 10 days and did not differ much during the rest of the storage time, therefore, we could assume that the specific surface area in these two emulsion samples were the same during the initiation stage of the oxidation (**Figure 12 & 13**). The retarded lipid oxidation was mostly attributed to a stronger protection of the interface against the interaction with pro-oxidants such as transition metals or against the adsorption of surface active oxidation products produced during the lipid oxidation. Kellerby et al. [2006] reported in their study in which post-emulsification cross-linking of CN was performed using transglutaminase as the catalyst that the cross-linking occurred preferably between the adsorbed proteins rather than that between the adsorbed and non-adsorbed proteins. Thus only the cohesiveness of the interfacial CN was increased and the thickness was assumed to be unchanged. As a result, the cross-linking did not influence the diffusion of small pro-oxidant molecules to the core of oil droplets and the oxidative stability of the cross-linked emulsion was almost the same as that of the uncross-linked one. Partanen et al. [2013] studied the transglutaminase catalyzed cross-linking of β-casein at an O/W interface and found an increased density and decreased thickness (11–12 nm to 8–9 nm) of the interfacial protein layer due to the intramolecular cross-linking. In our study, CN was cross-linked prior to the emulsification process, the polymerized protein could form a thicker and denser interfacial protein layer and thus increase the amount of anti-oxidative groups located at the interface and provide a stronger barrier against competitive adsorption by oil oxidation products. Based on this pre-emulsification cross-linking of protein emulsifiers might be considered a better option to make emulsions with better oxidative stability.

5. Conclusions and future perspectives

In this study, two major components of milk proteins, CN and WPI, were modified by different approaches in order to enhance their functionality as emulsifiers. Succinylation and EDA modification enhanced the electrostatic repulsion between droplets particularly at pH 4–7. Vanillic acid modification improved the reactivity of WPI towards laccase whereafter droplet aggregation was prevented by post-emulsification cross-linking. Pre-emulsification of CN by Tgase retarded the rate of lipid oxidation in the emulsion.

To the highest extent, the succinylation shifted the pI of CN from 4.2 to 2.7, and the EDA modification shifted the pI of CN and WPI from 4.2 to 9.4 and from 4.9 to 9.5 respectively. CN and WPI showed different reactivity to the EDA modification depending on their structure and solubility at their isoelectric points. By changing the ratio of chemical modifiers and the protein, the extent of the modifications could be controlled. MALDI-TOF method was used for the first time to characterize the chemically modified proteins and was proven to be a very powerful tool to calculate the precise number of the modified amino acids. The modified CN and WPI were found to have better emulsifying properties compared to their unmodified counterparts at certain pH range. In particular, the EDA modification, as an additional approach to esterification methods, was probably more effective since the modified proteins were able to stabilize an emulsion through the acidic and neutral pH range. Since chemical modifications have been used to change the net charge or hydrophilic-lipophilic balance (HLB) value of surfactant emulsifiers to improve their emulsifying properties in various products, the succinylation and the EDA modification indicate the possibility to expand the use of protein emulsifiers to emulsions at a broader range of pH by altering their isoelectric points. The study of these two types of modifications gives a good reference on controlling the droplet surface charge to optimize the emulsion stability at a given pH.

Enzymatic modifications, laccase and Tgase catalyzed cross-linking of milk proteins, were also studied in the present work. Again, the reactivity of CN and WPI towards the enzymes was found to be dependent on their structure and the accessibility of the reactive sites. WPI, in its native state, was proven not to be a good substrate for either laccase or Tgase. Incorporating artificial reactive sites onto the surface of WPI significantly improved its reactivity to laccase, and a number of pre-treatments have been reported to make WPI more susceptible to Tgase. The succinylated or EDA modified proteins could be very interesting

substrate to Tgase. Since in a fully modified protein, all the amino groups could be converted to carboxyl groups or the other way around, it might give a chance to tailor the cross-linking between a succinylated or EDA modified protein and another unmodified one.

Based on a good reactivity of CN towards Tgase or the vanillic acid modified WPI towards laccase, enzymatic cross-linking was applied before and after the emulsification process and the effect of cross-linking on emulsion stability were investigated. The pre-emulsification cross-linking of CN built a stronger protective layer at the O/W interface against lipid oxidation which could chemically impair the stability of an emulsion. On the other hand, the post-emulsification cross-linking of Van-WPI significantly improved the steric repulsion against droplet aggregation most likely due to a denser or more viscoelastic interfacial layer.

The results presented in this study have implied a great opportunity to improve the stability or functionality of milk proteins via chemical or enzymatic modifications. The alteration of pI of milk protein itself might also open a new path to stabilize milk proteins in dairy products at acidic or neutral pH. However, chemical modifications applied on protein ingredients in foods might raise safety issues. As a continuation of this study, it is worthwhile looking for food grade (enzyme based) modification methods that can achieve similar properties as the chemically modified proteins. On the other hand, it would also be interesting to test these modification methods on non-protein emulsifiers for non-food applications.

Some explanation on the effect of enzymatic cross-linking on emulsion stability in this study was based on the conclusions or hypothesis made in previous relevant studies. The exact mechanism of stabilization of emulsions by the modified proteins was still unclear. Therefore, it is of great importance to investigate the interfacial properties of the modified proteins, for example, the thickness of the interfacial protein layer, the interfacial tension and the rheological properties. From an application perspective, the second part of this study indicated a potential to improve the oxidative stability of the droplets via enzymatic modification on the interfacial protein structure. For example, it would be interesting to cross-link the protein moiety of gum arabic in citrus oil emulsions which are sensitive to the oxidation of limonene. Investigations on other non-chemical approaches to improve the susceptibility of whey proteins to a cross-linking enzyme would be necessary.

References

Agboola, S.O. & Dalgleish, D.G. 1996. Enzymatic hydrolysis of milk proteins used for emulsion formation. 2. effects of calcium, pH, and ethanol on the stability of the emulsions. Journal of Agricultural and Food Chemistry, Vol. 44, No. 11, pp. 3637-3642.

Ahmed, K., Li, Y., McClements, D.J. & Xiao, H. 2012. Nanoemulsion- and emulsion-based delivery systems for curcumin: Encapsulation and release properties. Food Chemistry, Vol. 132, No. 2, pp. 799-807.

Aminlari, M., Ramezani, R. & Jadidi, F. 2005. Effect of Maillard-based conjugation with dextran on the functional properties of lysozyme and casein. Journal of the science of food and agriculture, Vol. 85, No. 15, pp. 2617-2624.

Araújo, E.S. & de Oliveira, H.P. 2011. Phase inversions in emulsions probed by electrical impedance spectroscopy. Journal of Dispersion Science and Technology, Vol. 32, No. 11, pp. 1649-1654.

Augustin, M.A., Sanguansri, L. & Bode, O. 2006. Maillard reaction products as encapsulants for fish oil powders. Journal of Food Science, Vol. 71, No. 2, pp. E25-E32.

Bönisch, M.P., Lauber, S. & Kulozik, U. 2004. Effect of ultra-high temperature treatment on the enzymatic cross-linking of micellar casein and sodium caseinate by transglutaminase. Journal of Food Science, Vol. 69, No. 8, pp. E398-E404.

Bos, M.A. & van Vliet, T. 2001. Interfacial rheological properties of adsorbed protein layers and surfactants: a review. Advances in Colloid and Interface Science, Vol. 91, No. 3, pp. 437-471.

Bourbonnais, R. & Paice, M.G. 1990. Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation. FEBS letters, Vol. 267, No. 1, pp. 99-102.

Boyd, J.V., Mitchell, J.R., Irons, L., Musselwhite, P.R. & Sherman, P. 1973. The mechanical properties of milk protein films spread at the air-water interface. Journal of Colloid and Interface Science, Vol. 45, No. 3, pp. 478-486.

Brown, K.C., Yang, S.-. & Kodadek, T. 1995. Highly specific oxidative cross-linking of proteins mediated by a nickel - peptide complex. Biochemistry, Vol. 34, No. 14, pp. 4733-4739.

Brownlow, S., Morais Cabral, J.H., Cooper, R., Flower, D.R., Yewdall, S.J., Polikarpov, I., North, A.C.T. & Sawyer, L. 1997. Bovine β-lactoglobulin at 1.8 Å resolution - Still an enigmatic lipocalin. Structure, Vol. 5, No. 4, pp. 481-495.

Buchert, J., Ercili Cura, D., Ma, H., Gasparetti, C., Monogioudi, E., Faccio, G., Mattinen, M., Boer, H., Partanen, R., Selinheimo, E., Lantto, R. & Kruus, K. 2010. Crosslinking food proteins for improved functionality. Annual Review of Food Science and Technology, Vol. 1, pp. 113-138.

Caessens, P.W.J.R., Visser, S., Gruppen, H. & Voragen, A.G.J. 1999. β-Lactoglobulin hydrolysis. 1. Peptide composition and functional properties of hydrolysates obtained by the action of plasmin, trypsin, and *Staphylococcus aureus* V8 protease. Journal of Agricultural and Food Chemistry, Vol. 47, No. 8, pp. 2973-2979.

Capek, I. 2004. Degradation of kinetically-stable o/w emulsions. Advances in Colloid and Interface Science, Vol. 107, No. 2–3, pp. 125-155.

Chanamai, R. & McClements, D.J. 2000. Impact of weighting agents and sucrose on gravitational separation of beverage emulsions. Journal of Agricultural and Food Chemistry, Vol. 48, No. 11, pp. 5561-5565.

Couper, A. 1993. Surface tension and its measurement. In: B. W, Rossiter and R. C. Baetzold (Ed.), Physical Methods of Chemistry, 2nd ed. New York: Wiley-Interscience. Vol. 9 A.

Coupland, J.N. & McClements, D.J. 2001. Droplet size determination in food emulsions: Comparison of ultrasonic and light scattering methods. Journal of Food Engineering, Vol. 50, No. 2, pp. 117-120.

Coupland, J.N. & McClements, D.J. 1996. Lipid oxidation in food emulsions. Trends in Food Science and Technology, Vol. 7, No. 3, pp. 83-91.

Crenwelge, D.D., Dill, C.W., Tybor, P.T. & Landmann, W.A. 1974. A comparison of the emulsification capacities of some protein concentrates. Journal of Food Science, Vol. 39, No. 1, pp. 175-177.

Dalgleish, D.G. 1993. The sizes and conformations of the proteins in adsorbed layers of individual caseins on latices and in oil-in-water emulsions. Colloids and Surfaces B: Biointerfaces, Vol. 1, No. 1, pp. 1-8.

Damodaran, S. 1996. Amino acids, peptides, and proteins. In: O.R. Fennema (Ed.), Food Chemistry, 3rd ed. New York: Marcel Dekker. pp. 321-429.

Day, L., Xu, M., Lundin, L. & Wooster, T.J. 2009. Interfacial properties of deamidated wheat protein in relation to its ability to stabilise oil-in-water emulsions. Food Hydrocolloids, Vol. 23, No. 8, pp. 2158-2167.

Dickinson, E. 2009. Hydrocolloids as emulsifiers and emulsion stabilizers. Food Hydrocolloids, Vol. 23, No. 6, pp. 1473-1482.

Dickinson, E. 1998a. Proteins at interfaces and in emulsions. Stability, rheology and interactions. Journal of the Chemical Society - Faraday Transactions, Vol. 94, No. 12, pp. 1657-1669.

Dickinson, E. 1998b. Structure, stability and rheology of flocculated emulsions. Current Opinion in Colloid and Interface Science, Vol. 3, No. 6, pp. 633-638.

Dickinson, E. 1997. Properties of Emulsions Stabilized with Milk Proteins: Overview of Some Recent Developments. Journal of Dairy Science, Vol. 80, No. 10, pp. 2607-2619.

Dickinson, E. 1993. Towards more natural emulsifiers. Trends in Food Science and Technology, Vol. 4, No. 10, pp. 330-334.

Dickinson, E. & McClements, D.J. 1995. Advances in Food Colloids. London: Blackie.

Dickinson, E. & Yamamoto, Y. 1996. Rheology of milk protein gels and protein-stabilized emulsion gels cross-linked with transglutaminase. Journal of Agricultural and Food Chemistry, Vol. 44, No. 6, pp. 1371-1377.

Dickinson, E. 2009. Hydrocolloids as emulsifiers and emulsion stabilizers. Food Hydrocolloids, Vol. 23, No. 6, pp. 1473-1482.

Dickinson, E., Ritzoulis, C., Yamamoto, Y. & Logan, H. 1999. Ostwald ripening of protein-stabilized emulsions: effect of transglutaminase crosslinking. Colloids and Surfaces B: Biointerfaces, Vol. 12, No. 3–6, pp. 139-146.

Donnelly, J.L., Decker, E.A. & McClements, D.J. 1998. Iron-catalyzed oxidation of Menhaden oil as affected by emulsifiers. Journal of Food Science, Vol. 63, No. 6, pp. 997-1000.

Elias, R.J., Kellerby, S.S. & Decker, E.A. 2008. Antioxidant activity of proteins and peptides. Critical Reviews in Food Science and Nutrition, Vol. 48, No. 5, pp. 430-441.

Ercili Cura, D., Lantto, R., Lille, M., Andberg, M., Kruus, K. & Buchert, J. 2009. Laccase-aided protein modification: Effects on the structural properties of acidified sodium caseinate gels. International Dairy Journal, Vol. 19, No. 12, pp. 737-745.

Ercili-Cura, D., Partanen, R., Husband, F., Ridout, M., Macierzanka, A., Lille, M., Boer, H., Lantto, R., Buchert, J. & Mackie, A.R. 2012. Enzymatic cross-linking of β-lactoglobulin in solution and at air–water interface: Structural constraints. Food Hydrocolloids, Vol. 28, No. 1, pp. 1-9.

Færgemand, M., Murray, B.S. & Dickinson, E. 1997. Cross-linking of milk proteins with transglutaminase at the oil−water interface. Journal of Agricultural and Food Chemistry, Vol. 45, No. 7, pp. 2514-2519.

Færgemand, M., Murray, B.S., Dickinson, E. & Qvist, K.B. 1999. Cross-linking of adsorbed casein films with transglutaminase. International Dairy Journal, Vol. 9, No. 3-6, pp. 343-346.

Færgemand, M., Otte, J. & Qvist, K.B. 1998a. Cross-Linking of Whey Proteins by Enzymatic Oxidation. Journal of Agricultural and Food Chemistry, Vol. 46, No. 4, pp. 1326-1333.

Færgemand, M., Otte, J. & Qvist, K.B. 1998b. Emulsifying properties of milk proteins cross-linked with microbial transglutaminase. International Dairy Journal, Vol. 8, No. 8, pp. 715-723.

Ferrando, M. & Spiess, W.E.L. 2000. Review: Confocal scanning laser microscopy. A powerful tool in food science. Food Science and Technology International, Vol. 6, No. 4, pp. 267-284.

Figueroa-Espinoza, M.C., Morel, M.H. & Rouau, X. 1998. Effect of lysine, tyrosine, cysteine, and glutathione on the oxidative cross-Linking of feruloylated arabinoxylans by a fungal laccase. Journal of Agricultural and Food Chemistry, Vol. 46, No. 7, pp. 2583-2589.

Flanagan, J., Gunning, Y. & FitzGerald, R.J. 2003. Effect of cross-linking with transglutaminase on the heat stability and some functional characteristics of sodium caseinate. Food Research International, Vol. 36, No. 3, pp. 267-274.

Flores, I., Cabra, V., Quirasco, M.C., Farres, A. & Galvez, A. 2010. Emulsifying properties of chemically deamidated corn (Zea Mays) gluten meal. Food Science and Technology International, Vol. 16, No. 3, pp. 241-250.

Folk, J.E. & Finlayson, J.S. 1977. The ε-(γ-glutamyl)lysine crosslink and the catalytic role of transglutaminases. Advances in Protein Chemistry, Vol. 31, pp. 1-33.

Frankel, E.N., Huang, S.W., Kanner, J. & German, J.B. 1994. Interfacial phenomena in the evaluation of antioxidants: Bulk oils vs emulsions. Journal of Agricultural and Food Chemistry, Vol. 42, No. 5, pp. 1054-1059.

Frasconi, M., Favero, G., Boer, H., Koivula, A. & Mazzei, F. 2010. Kinetic and biochemical properties of high and low redox potential laccases from fungal and plant origin. Biochimica et Biophysica Acta - Proteins and Proteomics, Vol. 1804, No. 4, pp. 899-908.

Friedman, M. 1999. Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. Journal of Agricultural and Food Chemistry, Vol. 47, No. 4, pp. 1295-1319.

Gallegos, C. & Franco, J.M. 1999. Rheology of food, cosmetics and pharmaceuticals. Current Opinion in Colloid & Interface Science, Vol. 4, No. 4, pp. 288-293.

Goff, H.D. & Hill, A.R. 1993. Chemistry and physics. In: Y.H. Hui (ed.), Dairy Science and Technology Handbook, New York: VCH Publishers. Vol. 1, pp. 1-82.

Gohtani, S., Sirendi, M., Yamamoto, N., Kajikawa, K. & Yamano, Y. 1999. Effect of droplet size on oxidation of docosahexaenoic acid in emulsion system. Journal of Dispersion Science and Technology, Vol. 20, No. 5, pp. 1319-1325.

Greenwood, R. & Kendall, K. 1999. Selection of suitable dispersants for aqueous suspensions of zirconia and titania powders using acoustophoresis. Journal of the European Ceramic Society, Vol. 19, No. 4, pp. 479-488.

Groninger, H.S. 1973. Preparation and properties of succinylated fish myofibrillar protein. Journal of Agricultural and Food Chemistry, Vol. 21, No. 6, pp. 978-981.

Guzey, D. & McClements, D.J. 2006. Formation, stability and properties of multilayer emulsions for application in the food industry. Advances in Colloid and Interface Science, Vol. 128-130, pp. 227-248.

Hall, T.C., McLeester, R.C. & Bliss, F.A. 1977. Equal expression of the maternal and paternal alleles for the polypeptide subunits of the major storage protein of the bean *Phaseolus vulgaris* L. Plant Physiol, Vol. 59, pp. 1122-1124.

Han, X.-Q. & Damodaran, S. 1996. Thermodynamic compatibility of substrate proteins affects their cross-linking by transglutaminase. Journal of Agricultural and Food Chemistry, Vol. 44, No. 5, pp. 1211-1217.

Hattori, M., Numamoto, K., Kobayashi, K. & Takahashi, K. 2000. Functional changes in β-lactoglobulin by conjugation with cationic saccharides. Journal of Agricultural and Food Chemistry, Vol. 48, No. 6, pp. 2050-2056.

Havea, P., Watkinson, P. & Kuhn-Sherlock, B. 2009. Heat-induced whey protein gels: Protein-protein interactions and functional properties. Journal of Agricultural and Food Chemistry, Vol. 57, No. 4, pp. 1506-1512.

Henderson, D.E., Slickman, A.M. & Henderson, S.K. 1999. Quantitative HPLC determination of the antioxidant activity of capsaicin on the formation of lipid hydroperoxides of linoleic acid: A comparative study against BHT and melatonin. Journal of Agricultural and Food Chemistry, Vol. 47, No. 7, pp. 2563-2570.

Hiller, B. & Lorenzen, P.C. 2009. Functional properties of milk proteins as affected by enzymatic oligomerisation. Food Research International, Vol. 42, No. 8, pp. 899-908.

Hinz, K., Huppertz, T., Kulozik, U. & Kelly, A.L. 2007. Influence of enzymatic cross-linking on milk fat globules and emulsifying properties of milk proteins. International Dairy Journal, Vol. 17, No. 4, pp. 289-293.

Hoagland, P.D. 1968. Acylated β-caseins. Effect of alkyl group size on calcium ion sensitivity and on aggregation. Biochemistry, Vol. 7, No. 7, pp. 2542-2546.

Hu, M., McClements, D.J. & Decker, E.A. 2003. Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. Journal of Agricultural and Food Chemistry, Vol. 51, No. 6, pp. 1696-1700.

Hunter, R.J. 1989. Foundations of Colloid Science, Oxford: Oxford University Press. Vol. 1.

Husband, F.A., Wilde, P.J., Mackie, A.R. & Garrood, M.J. 1997. A comparison of the functional and interfacial properties of β-casein and dephosphorylated β-casein. Journal of Colloid and Interface Science, Vol. 195, No. 1, pp. 77-85.

Imai, H., Maeda, T., Shima, M. & Adachi, S. 2008. Oxidation of methyl linoleate in oil-in-water micro- and nanoemulsion systems. Journal of the American Oil Chemists' Society, Vol. 85, No. 9, pp. 809-815.

Jaros, D., Partschefeld, C., Henle, T. & Rohm, H. 2006. Transglutaminase in dairy products: Chemistry, physics, applications. Journal of Texture Studies, Vol. 37, No. 2, pp. 113-155.

Kellerby, S.S., Yeun, S.G., McClements, D.J. & Decker, E.A. 2006. Lipid oxidation in a menhaden oil-in-water emulsion stabilized by sodium caseinate cross-linked with transglutaminase. Journal of Agricultural and Food Chemistry, Vol. 54, No. 26, pp. 10222-10227.

Khalloufi, S., Corredig, M., Goff, H.D. & Alexander, M. 2009. Flaxseed gums and their adsorption on whey protein-stabilized oil-in-water emulsions. Food Hydrocolloids, Vol. 23, No. 3, pp. 611-618.

Khan, B.A., Akhtar, N., Khan, H.M.S., Waseem, K., Mahmood, T., Rasul, A., Iqbal, M. & Khan, H. 2011. Basics of pharmaceutical emulsions: A review. African Journal of Pharmacy and Pharmacology, Vol. 5, No. 25, pp. 2715-2725.

Kim, J.M., Whang, J.H. & Suh, H.J. 2004. Enhancement of angiotensin I converting enzyme inhibitory activity and improvement of the emulsifying and foaming properties of corn gluten hydrolysate using ultrafiltration membranes. European Food Research and Technology, Vol. 218, No. 2, pp. 133-138.

Kim, S. & Cavaco-Paulo, A. 2012. Laccase-catalysed protein–flavonoid conjugates for flax fibre modification. Applied Microbiology and Biotechnology, Vol. 93, No. 2, pp. 585-600.

Kirby, A.R., Gunning, A.P. & Morris, V.J. 1995. Atomic force microscopy in food research: A new technique comes of age. Trends in Food Science & Technology, Vol. 6, No. 11, pp. 359-365.

Kubow, S. 1992. Routes of formation and toxic consequences of lipid oxidation products in foods. Free Radical Biology and Medicine, Vol. 12, No. 1, pp. 63-81.

Lakkis, J. & Villota, R. 1990. Study on the foaming and emulsifying properties of whey protein hydrolysates. AIChE Symposium Series. Vol. 86. pp. 87.

Lantto, R., Puolanne, E., Kalkkinen, N., Buchert, J. & Autio, K. 2005. Enzyme-aided modification of chicken-breast myofibril proteins: effect of laccase and transglutaminase on gelation and thermal stability. Journal of Agricultural and Food Chemistry, Vol. 53, No. 23, pp. 9231-9237.

Lantto, R., Puolanne, E., Katina, K., Niemistö, M., Buchert, J. & Autio, K. 2007. Effect of laccase and transglutaminase on the textural and water-binding properties of cooked chicken breast meat gels. European Food Research and Technology, Vol. 225, No. 1, pp. 75-83.

Lantto, R., Schönberg, C., Buchert, J. & Heine, E. 2004. Effects of laccase-mediator combinations on wool. Textile Research Journal, Vol. 74, No. 8, pp. 713-717.

Lawal, O.S. & Adebowale, K.O. 2004. Effect of acetylation and succinylation on solubility profile, water absorption capacity, oil absorption capacity and emulsifying properties of mucuna bean (*Mucuna pruriens*) protein concentrate. Nahrung - Food, Vol. 48, No. 2, pp. 129-136.

Li, Y., Zheng, J., Xiao, H. & McClements, D.J. 2012. Nanoemulsion-based delivery systems for poorly water-soluble bioactive compounds: Influence of formulation

parameters on polymethoxyflavone crystallization. Food Hydrocolloids, Vol. 27, No. 2, pp. 517-528.

Liu, M. & Damodaran, S. 1999. Effect of transglutaminase-catalyzed polymerization of β-casein on its emulsifying properties. Journal of Agricultural and Food Chemistry, Vol. 47, No. 4, pp. 1514-1519.

Losada-Barreiro, S., Sánchez-Paz, V., Bravo-Díaz, C., Paiva-Martins, F. & Romsted, L.S. 2012. Temperature and emulsifier concentration effects on gallic acid distribution in a model food emulsion. Journal of Colloid and Interface Science, Vol. 370, No. 1, pp. 73-79.

Mancuso, J.R., McClements, D.J. & Decker, E.A. 1999. The effects of surfactant type, pH, and chelators on the oxidation of salmon oil-in-water emulsions. Journal of Agricultural and Food Chemistry, Vol. 47, No. 10, pp. 4112-4116.

Marshall, W.H., Dutson, T.R., Carpenter, Z.L. & Smith, G.C. 1975. A simple method for emulsion end-point determinations. Journal of Food Science, Vol. 40, No. 4, pp. 896-897.

Mat Easa, A., Hill, S.E., Mitchell, J.R. & Taylor, A.J. 1996. Bovine serum albumin gelation as a result of the Maillard reaction. Food Hydrocolloids, Vol. 10, No. 2, pp. 199-202.

Matemu, A.O., Kayahara, H., Murasawa, H., Katayama, S. & Nakamura, S. 2011. Improved emulsifying properties of soy proteins by acylation with saturated fatty acids. Food Chemistry, Vol. 124, No. 2, pp. 596-602.

Matheis, G. & Whitaker, J.R. 1984. Peroxidase-catalyzed cross linking of proteins. Journal of Protein Chemistry, Vol. 3, No. 1, pp. 35-48.

Mattarella, N.L. & Richardson, T. 1983. Physicochemical and functional properties of positively charged derivatives of bovine β-lactoglobulin. Journal of Agricultural and Food Chemistry, Vol. 31, No. 5, pp. 972-978.

Mattinen, M., Kruus, K., Buchert, J., Nielsen, J.H., Andersen, H.J. & Steffensen, C.L. 2005. Laccase-catalyzed polymerization of tyrosine-containing peptides. FEBS Journal, Vol. 272, No. 14, pp. 3640-3650.

McClements, D.J. 2010. Emulsion design to improve the delivery of functional lipophilic components. Annual Review of Food Science and Technology, Vol. 1, pp. 241-269.

McClements, D.J. 2004. Food Emulsions: Principles, Practices, and Techniques, 2nd ed. Boca Raton: CRC Press.

McClements, D.J. & Decker, E.A. 2000. Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. Journal of Food Science, Vol. 65, No. 8, pp. 1270-1282.

McDonald, R.E. & Hultin, H.O. 1987. Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. Journal ofFood Science, Vol. 52, No. 1, pp. 15-21.

Miller, R., Ferri, J.K., Javadi, A., Krägel, J., Mucic, N. & Wüstneck, R. 2010. Rheology of interfacial layers. Colloid and Polymer Science, Vol. 288, No. 9, pp. 937-950.

Mitidieri, F.E. & Wagner, J.R. 2002. Coalescence of o/w emulsions stabilized by whey and isolate soybean proteins. Influence of thermal denaturation, salt addition and competitive interfacial adsorption. Food Research International, Vol. 35, No. 6, pp. 547-557.

Monogioudi, E., Creusot, N., Kruus, K., Gruppen, H., Buchert, J. & Mattinen, M. 2009. Cross-linking of β-casein by *Trichoderma reesei* tyrosinase and *Streptoverticillium mobaraense* transglutaminase followed by SEC–MALLS. Food Hydrocolloids, Vol. 23, No. 7, pp. 2008-2015.

Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J. & Mattinen, M. 2011. Effect of enzymatic cross-linking of β-casein on proteolysis by pepsin. Food Hydrocolloids, Vol. 25, No. 1, pp. 71-81.

Moro, A., Gatti, C. & Delorenzi, N. 2001. Hydrophobicity of whey protein concentrates measured by fluorescence quenching and its relation with surface functional properties. Journal of Agricultural and Food Chemistry, Vol. 49, No. 10, pp. 4784-4789.

Motoki, M. & Seguro, K. 1998. Transglutaminase and its use for food processing. Trends in Food Science and Technology, Vol. 9, No. 5, pp. 204-210.

Mühlrad, A., Corsi, A. & Granata, A.L. 1968. Studies on the properties of chemically modified actin. I. Photooxidation, succinylation, nitration. Biochimica et Biophysica Acta, Vol. 162, No. 3, pp. 435-443.

Murray, B.S. 2002. Interfacial rheology of food emulsifiers and proteins. Current Opinion in Colloid & Interface Science, Vol. 7, No. 5-6, pp. 426-431.

Mustafa, R., Muniglia, L., Rovel, B. & Girardin, M. 2005. Phenolic colorants obtained by enzymatic synthesis using a fungal laccase in a hydro-organic biphasic system. Food Research International, Vol. 38, No. 8-9, pp. 995-1000.

Nakai, S. & Li-Chan, E. 1988. In: Hydrophobic Interactions in Food Systems, Boca Raton: CRC Press. pp. 145-151.

Nakaya, K., Ushio, H., Matsukawa, S., Shimizu, M. & Ohshima, T. 2005. Effects of droplet size on the oxidative stability of oil-in-water emulsions. Lipids, Vol. 40, No. 5, pp. 501-507.

Nawar, W.W. 1996. Lipids. In: O. R. Fennema (Ed.), Food Chemistry, New York: Marcel Dekker. pp. 225-319.

Neirynck, N., Van der Meeren, P., Bayarri Gorbe, S., Dierckx, S. & Dewettinck, K. 2004. Improved emulsion stabilizing properties of whey protein isolate by conjugation with pectins. Food Hydrocolloids, Vol. 18, No. 6, pp. 949-957.

Neirynck, N., Van der Meeren, P., Lukaszewicz-Lausecker, M., Cocquyt, J., Verbeken, D. & Dewettinck, K. 2007. Influence of pH and biopolymer ratio on

whey protein-pectin interactions in aqueous solutions and in O/W emulsions. Colloids and Surfaces A: Physicochemical and Engineering Aspects, Vol. 298, No. 1-2, pp. 99-107.

Oliver, C.M., Melton, L.D. & Stanley, R.A. 2006. Functional properties of caseinate glycoconjugates prepared by controlled heating in the 'dry' state. Journal of the science of food and agriculture, Vol. 86, No. 5, pp. 732-740.

Osborn, H.T. & Akoh, C.C. 2004. Effect of emulsifier type, droplet size, and oil concentration on lipid oxidation in structured lipid-based oil-in-water emulsions. Food Chemistry, Vol. 84, No. 3, pp. 451-456.

Panyam, D. & Kilara, A. 1996. Enhancing the functionality of food proteins by enzymatic modification. Trends in Food Science and Technology, Vol. 7, No. 4, pp. 120-125.

Paquot, C. & Hautfenne, A. 1987. Standard Methods for the Analysis of Oils, Fats and Derivatives, 7th ed. London: Blackwell.

Partanen, R., Forssell, P., Mackie, A. & Blomberg, E. 2013. Interfacial cross-linking of β-casein changes the structure of the adsorbed layer. Food Hydrocolloids, Vol. 32, No. 2, pp. 271-277.

Pearce, K.N. & Kinsella, J.E. 1978. Emulsifying properties of proteins: Evaluation of a turbidimetric technique. Journal of Agricultural and Food Chemistry, Vol. 26, No. 3, pp. 716-723.

Pelegrine, D.H.G. & Gasparetto, C.A. 2005. Whey proteins solubility as function of temperature and pH. Lebensmittel-Wissenschaft und-Technologie, Vol. 38, No. 1, pp. 77-80.

Perrechil, F.A. & Cunha, R.L. 2010. Oil-in-water emulsions stabilized by sodium caseinate: Influence of pH, high-pressure homogenization and locust bean gum addition. Journal of Food Engineering, Vol. 97, No. 4, pp. 441-448.

Robins, M.M., Watson, A.D. & Wilde, P.J. 2002. Emulsions - Creaming and rheology. Current Opinion in Colloid and Interface Science, Vol. 7, No. 5-6, pp. 419-425.

Rodríguez Couto, S. & Toca Herrera, J.L. 2006. Industrial and biotechnological applications of laccases: A review. Biotechnology Advances, Vol. 24, No. 5, pp. 500-513.

Rodriguez-Nogales, J.M. 2006. Effect of preheat treatment on the transglutaminase-catalyzed cross-linking of goat milk proteins. Process Biochemistry, Vol. 41, No. 2, pp. 430-437.

Romoscanu, A.I. & Mezzenga, R. 2005. Cross linking and rheological characterization of adsorbed protein layers at the oil-water interface. Langmuir, Vol. 21, No. 21, pp. 9689-9697.

Rysakov, V.M. & Rejmund, F. 2008. Limiting possibilities of the analysis of the microparticle parameters in the emulsions by the light scattering methods. Journal of Quantitative Spectroscopy and Radiative Transfer, Vol. 109, No. 7, pp. 1151-1161.

Salis, A., Bostrom, M., Medda, L., Cugia, F., Barse, B., Parsons, D.F., Ninham, B.W. & Monduzzi, M. 2011. Measurements and theoretical interpretation of points of zero charge/potential of BSA protein. Langmuir, Vol. 27, No. 18, pp. 11597-11604.

Schultz, S., Wagner, G., Urban, K. & Ulrich, J. 2004. High-pressure homogenization as a process for emulsion formation. Chemical Engineering and Technology, Vol. 27, No. 4, pp. 361-368.

Selinheimo, E., Lampila, P., Mattinen, M.L. & Buchert, J. 2008. Formation of protein-oligosaccharide conjugates by laccase and tyrosinase. Journal of Agricultural and Food Chemistry, Vol. 56, No. 9, pp. 3118-3128.

Sharma, R., Zakora, M. & Qvist, K.B. 2002. Characteristics of oil-water emulsions stabilised by an industrial α-lactalbumin concentrate, cross-linked before and after emulsification, by a microbial transglutaminase. Food Chemistry, Vol. 79, No. 4, pp. 493-500.

Sharma, S.K. & Dalgleish, D.G. 1993. Interactions between milk serum proteins and synthetic fat globule membrane during heating of homogenized whole milk. Journal of Agricultural and Food Chemistry, Vol. 41, No. 9, pp. 1407-1412.

Sherman, P. 1970. Rheology of Dispersed Systems. In: Industrial Rheology. London: Academic Press Inc. pp. 97-183.

Shirashoji, N., Jaeggi, J.J. & Lucey, J.A. 2006. Effect of trisodium citrate concentration and cooking time on the physicochemical properties of pasteurized process cheese. Journal of Dairy Science, Vol. 89, No. 1, pp. 15-28.

Silvestre, M.P.C., Chaiyasit, W., Brannan, R.G., McClements, D.J. & Decker, E.A. 2000. Ability of surfactant headgroup size to alter lipid and antioxidant oxidation in oil-in-water emulsions. Journal of Agricultural and Food Chemistry, Vol. 48, No. 6, pp. 2057-2061.

Singh, A.M. & Dalgleish, D.G. 1998. The Emulsifying Properties of Hydrolyzates of Whey Proteins. Journal of Dairy Science, Vol. 81, No. 4, pp. 918-924.

Singh, H. 1991. Modification of food proteins by covalent crosslinking. Trends in Food Science and Technology, Vol. 2, No. 2, pp. 196-200.

Sitohy, M., Chobert, J.M. & Haertlé, T. 2001. Improvement of solubility and of emulsifying properties of milk proteins at acid pHs by esterification. Nahrung - Food, Vol. 45, No. 2, pp. 87-93.

Smart, M.G., Fulcher, R.G. & Pechak, D.G. 1995. Recent Developments in the Microstructural Characterization of Foods. In: A.G. Gaonkar (Ed.), Characterization of Food. Amsterdam: Elsevier Science B.V. pp. 233-275.

Solans, C., Izquierdo, P., Nolla, J., Azemar, N. & Garcia-Celma, M.J. 2005. Nano-emulsions. Current Opinion in Colloid and Interface Science, Vol. 10, No. 3-4, pp. 102-110.

Stachel, S.J., Habeeb, R.L. & Van Vranken, D.L. 1996. Formation of constrained, fluorescent peptides via tryptophan dimerization and oxidation. Journal of the American Chemical Society, Vol. 118, No. 5, pp. 1225-1226.

Stauffer, C. 1999. Food emulsifiers. In: Emulsifier: Practical Guides for the Food Indulstry. St. Paul: Eagan Press. pp. 39-41.

Steger, P.J.K. & Mühlebach, S.F. 1998. Lipid peroxidation of IV lipid emulsions in TPN bags: The influence of tocopherols. Nutrition, Vol. 14, No. 2, pp. 179-185.

Stevenson, E.M., Horne, D.S. & Leaver, J. 1997. Displacement of native and thiolated β-casein from oil-water interfaces - Effect of heating, ageing and oil phase. Food Hydrocolloids, Vol. 11, No. 1, pp. 3-6.

Stevenson, R.J., Chen, X.D. & Mills, O.E. 1996. Modern analyses and binding studies of flavour volatiles with particular reference to dairy protein products. Food Research International, Vol. 29, No. 3-4, pp. 265-290.

Surh, J., Decker, E.A. & McClements, D.J. 2006. Influence of pH and pectin type on properties and stability of sodium-caseinate stabilized oil-in-water emulsions. Food Hydrocolloids, 7, Vol. 20, No. 5, pp. 607-618.

Swift, C.E., Lockett, C. & Fryer, P.J. 1961. The capacity of meat for emulsifying fat. Food Technology, Vol. 15, pp. 468-471.

Tang, C., Yang, X, Chen, Z., Wu, H. & Peng, Z. 2005. Physicochemical and structural characteristics of sodium caseinate biopolymers induced by microbial transglutaminase. Journal of Food Biochemistry, Vol. 29, No. 4, pp. 402-421.

Tavano, O.L. 2013. Protein hydrolysis using proteases: An important tool for food biotechnology. Journal of Molecular Catalysis B: Enzymatic, 6, Vol. 90, pp. 1-11.

Taylor, P. 1998. Ostwald ripening in emulsions. Advances in Colloid and Interface Science, Vol. 75, No. 2, pp. 107-163.

Tcholakova, S., Denkov, N.D., Ivanov, I.B. & Campbell, B. 2006. Coalescence stability of emulsions containing globular milk proteins. Advances in Colloid and Interface Science, Vol. 123-126, pp. 259-293.

Tcholakova, S., Denkov, N.D. & Lips, A. 2008. Comparison of solid particles, globular proteins and surfactants as emulsifiers. Physical Chemistry Chemical Physics, Vol. 10, No. 12, pp. 1608-1627.

Tcholakova, S., Denkov, N.D., Sidzhakova, D. & Campbell, B. 2006. Effect of thermal treatment, ionic strength, and pH on the short-term and long-term coalescence stability of β-Lactoglobulin Emulsions. Langmuir, Vol. 22, No. 14, pp. 6042-6052.

Thorpe, C., Hoober, K.L., Raje, S., Glynn, N.M., Burnside, J., Turi, G.K. & Coppock, D.L. 2002. Sulfhydryl oxidases: Emerging catalysts of protein disulfide bond formation in eukaryotes. Archives of Biochemistry and Biophysics, Vol. 405, No. 1, pp. 1-12.

Thurston, C.F. 1994. The structure and function of fungal laccases. Microbiology, Vol. 140, No. 1, pp. 19-26.

Turgeon, S.L., Gauthier, S.F. & Paquin, P. 1991. Interfacial and emulsifying properties of whey peptide fractions obtained with a two-step ultrafiltration process. Journal of Agricultural and Food Chemistry, Vol. 39, No. 4, pp. 673-676.

Uy, R. & Wold, F. 1977. Introduction of artificial crosslinks into proteins. Advances in Experimental Medicine and Biology, Vol. 86 A, pp. 169-186.

Van Hekken, D.L., Strange, E.D. & Lu, D.P. 1996. Functional properties of chemically phosphorylated whole casein. Journal of Dairy Science, Vol. 79, No. 11, pp. 1942-1949.

Walstra, P. 1999. Casein sub-micelles: Do they exist? International Dairy Journal, Vol. 9, No. 3-6, pp. 189-192.

Wierenga, P.A., Egmond, M.R., Voragen, A.G. & de Jongh, H.H. 2006. The adsorption and unfolding kinetics determines the folding state of proteins at the air-water interface and thereby the equation of state. Journal of Colloid and Interface Science, Vol. 299, No. 2, pp. 850-857.

Woo, S.L. & Richardson, T. 1983. Functional properties of phosphorylated β-lactoglobulin. Journal of Dairy Science, Vol. 66, No. 5, pp. 984-987.

Wooster, T.J. & Augustin, M.A. 2006. β-Lactoglobulin–dextran Maillard conjugates: Their effect on interfacial thickness and emulsion stability. Journal of Colloid and Interface Science, Vol. 303, No. 2, pp. 564-572.

Wu, N., Huang, X., Yang, X., Guo, J., Zheng, E., Yin, S., Zhu, J., Qi, J., He, X. & Zhang, J. 2012. Stabilization of soybean oil body emulsions using ι-carrageenan: Effects of salt, thermal treatment and freeze-thaw cycling. Food Hydrocolloids, Vol. 28, No. 1, pp. 110-120.

Yaropolov, A.I., Skorobogat'ko, O.V., Vartanov, S.S. & Varfolomeyev, S.D. 1994. Laccase - Properties, catalytic mechanism, and applicability. Applied Biochemistry and Biotechnology, Vol. 49, No. 3, pp. 257-280.

Zhang, T., Jiang, B., Mu, W. & Wang, Z. 2009. Emulsifying properties of chickpea protein isolates: Influence of pH and NaCl. Food Hydrocolloids, Vol. 23, No. 1, pp. 146-152.

PUBLICATION I

Sodium caseinates with an altered isoelectric point as emulsifiers in oil/water systems

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Sodium Caseinates with an Altered Isoelectric Point As Emulsifiers in Oil/Water Systems

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Sodium caseinate was chemically modified in order to alter its isoelectric point (pI). Negatively charged carboxylic groups were introduced to lower the pI, and positively charged amino groups to achieve the opposite. Different chemical amino acid modification approaches were studied and the modified proteins were characterized using free amino group assays, SDS−PAGE, MALDI-TOF mass spectrometry, and zeta potential measurements. Oil-in-water emulsions were prepared using these modified caseinates. The pH stability behavior of the emulsions was monitored, and interestingly, the stability of the emulsion could be modulated through steering the pI of caseinate. Using different modified caseinates, it was possible to create emulsions that were stable in the acid, neutral, and alkaline regions of the pH spectrum. The stability behavior of the emulsions correlated well with the theoretical and experimentally determined pI values of the caseinates. Storage stability of emulsions was also studied at pH values around 7, and emulsions made of modified caseinates showed storage stability similar to that of unmodified caseinate emulsions.

KEYWORDS: Emulsion; stability; MALDI-TOF; sodium caseinate; amino acid modification

INTRODUCTION

Oil-in-water (O/W) emulsions are widely used in various industrial application areas such as in food products, cosmetics, and pharmaceuticals. In general, an O/W emulsion refers to a dispersion of oil in an aqueous phase. Because of the difference in density between oil and water, O/W emulsions are thermodynamically unstable, and phase separation occurs as a function of time. In order to solve this problem, surface-active emulsifiers are utilized to stabilize the oil−water interface, and as a result, the de-emulsification process is retarded. A good emulsifier should have a specific molecular structure with moieties attracted to the water and oil phases, a good solubility in the continuous phase, and capability to adsorb the dispersed droplets quickly and form a condensed film with subsequent reduction of the interfacial tension. The capability to increase the viscosity of the continuous phase of an emulsion is also one of the important characteristics of an effective emulsifier. In terms of practical issues, it should function at low concentration and be inexpensive, nontoxic, and safe to handle (*1*).

Proteins, such as casein and whey protein (*2*), are widely used as emulsifiers. They prefer to localize at the emulsion interface because of their amphoteric nature and thereby lower the surface tension. At the interface, the hydrophobic parts of the protein are in the oil phase, while the hydrophilic parts remain in the water phase, providing an electrostatic and steric repulsive force against coalescence or disproportionation (*3*). A drawback with proteins as emulsifiers is their low solubility due to low net charge at pH values close to their isoelectric points (pI), and as a result of this, their emulsifying activity in that pH range is considerably reduced (*4*). In the case of caseins with an average isoelectric point around 4.5 (*5*), reduction of emulsifying activity at pH values 4−5 has been a severe limitation in casein's applications in the acidic pH range (*6*).

Derivatization of amino acid moieties in proteins by chemical or enzymatic modification is an efficient way to change the physicochemical properties of proteins such as charge, hydrophilicity, and viscosity (*7*). For example, glutaraldehyde mediated cross-linking of β-casein endows the emulsion with enhanced stability against disproportionation (*8*). Glycolconjugates such as caseinate−maltodextrin (*6*, *9*) and casein modified with glucose, ribose, fructose, lactose, and fructo-oligosaccharide (*10*) have been reported with improved solubility at the pI of unmodified caseinate, and increased thickness of interfacial layer and a better emulsifying capability were observed. Modifications such as thiolation (*11*), phosphorylation (*12*), acetylation, and succinylation (*13*) have also been studied in terms of changing physicochemical properties of the proteins to optimize their use as emulsifiers.

Succinylation (**Scheme 1**) is one of the most common ways to acylate the amino group of proteins. In this reaction, the positively charged ε-amino groups of lysine residues are modified with negatively charged succinic acid groups through a covalent bond, resulting in a protein with a decreased pI toward the pK_a of the introduced succinic acid group.

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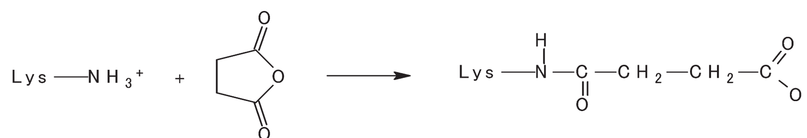
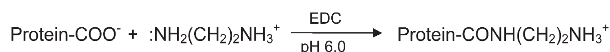
Scheme 1



Scheme 2



This modification has been applied to various proteins such as hydrolyzed soy proteins (*14*), soy (*15*), mung bean (*16*), fava bean legumin (*17*), lentil (*18*), leaf protein (*19*), peanut protein (*20*), and sunflower protein (*21*). Most of the succinylated products, compared with their native counterparts, exhibited improvement in functional properties such as increased solubility and better emulsifying capacity.

The ethylene diamine (EDA) modification (**Scheme 2**) alters the pI in the opposite way. Carboxyl groups in proteins react with EDA in the presence of a water-soluble carbodiimide, converting the negatively charged carboxyl groups into positively charged amino groups, resulting in a shift of the protein's pI toward the pK_a of an amino group in water (*22*).

So far, there has been no work published on using EDA-modified caseinate as an emulsifier. Ethylenediamine used for this type of modification might not be an ideal chemical for food emulsion applications, but it can be used as a model to validate the impact of an increased pI on emulsion stability.

In this work, physicochemical properties of caseinate were altered through succinylation and EDA modification in order to shift the isoelectric point toward both the acidic and the alkaline regions of the pH spectrum. By shifting the isoelectric point toward alkaline pH, it would possible to use caseinates as emulsifiers in the pH range from 3 to 7, which is an important pH range for food applications. In this study, the suitability of chemically modified sodium caseinates for this purpose were compared.

MATERIALS AND METHODS

Materials. Sodium caseinate was obtained from KasLink-Foods (Finland) (Protein 94%, Lactose 0.1%, Fat 1%, Ash 3.5%) and flaxseed oil was purchased from Elixi Oil Oy (Somero, Finland), where the fatty acid content was the following 4% 16:0, 3% 18:0, 12% 18:1, 15% 18:2, and 66% 18:3. Succinic anhydride (Purity ≥97.0%) and ethylenediamine dihydrochloride (Purity ≥99.0%) were purchased from Sigma. EDC (Purity ≥98.0%) was purchased from Pierce.

Modification of Na-Caseinate by Succinylation. A 3 mg/mL sodium caseinate solution was prepared by dissolving the protein in PBS buffer (pH 7.0), and the pH was adjusted to 8.0 using a 1.0 M NaOH solution. Succinic anhydride was added at a concentration of 0.03, 0.06, 0.1, 0.2, 0.3, 0.6, 1, and 4 g/g of protein, and 1.0 M NaOH was used to maintain the pH above 8.0 during the reaction. The mixture was stirred for 2 h at room temperature after all of the succinic anhydride was completely dissolved. Dialysis against distilled water was then applied to remove the unreacted reagents. Succinylated sodium caseinate was recovered by freeze-drying. The succinylated caseinate using 1 g of succinic anhydride per gram of sodium caseinate (SUCA) was made in large scale (> 300 mg) in order to be used as the emulsifier.

Modification of Na-Caseinate by EDA. A 3 mg/mL solution of sodium caseinate, which had been partially succinylated with 0.06 and 1 g of succinic anhydride per gram of protein was prepared by dissolving the protein in 0.1 M MES buffer containing 1 M ethylenediamine dihydrochloride. The pH was adjusted to 4.7 with a 0.1 M NaOH solution, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added at a concentration of 2 mg/mL. The reaction mixture was stirred for 2 h at room temperature. Dialysis against a 0.02 M NaH_2PO_4 and 0.15 M NaCl solution at pH 7.5 was used to quench the reaction, and dialysis against sterilized water was applied afterward to remove the unreacted reagents. The EDA modified caseinate was recovered by freeze-drying. The modification was also performed using bovine serum albumin (BSA) and whey protein as a comparison. EDA modified succinylated caseinate using 0.06 g of succinic anhydride per gram of sodium caseinate (EDCA) was made in large scale (> 300 mg) in order to be used as the emulsifier.

Analysis of the Extent of Modification. The 2,4,6-trinitrobenzene sulfonic acid (TNBSA) method of Hall et al. (*23*) was used to determine the extent of succinylation and EDA modification. The modified proteins and unmodified controls were dissolved in 0.1 M $NaHCO_3$ at a concentration of 200 μg/mL. After this, 0.25 mL of 0.01% solution of TNBSA was added to 0.5 mL of protein sample solution and mixed well. The samples were incubated at 37 °C for 2 h, and 0.25 mL of 10% SDS and 0.125 mL of 1 M HCl were added. The absorbance of the solutions was measured at 335 nm in a spectrophotometer (Perkin-Elmer Lambda 45 UV/vis Spectrometer, USA) against a reagent blank. The absorbance of the unmodified sodium caseinate was set equal to 100%. The relative percentage of modified lysine groups was obtained using the following formula:

$$\text{Percentage of modified lysine groups} = 1 - (\text{absorbance of modified caseinate}/\text{absorbance of unmodified caseinate}) \times 100\%$$

Matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) was applied to determine the extent of the chemical modifications. Sinapinic acid was selected as the matrix and dissolved to saturation in a 1:1 mixture of 0.1% trifluoroacetic acid (TFA) and acetonitrile. The samples of succinylated caseinate and EDA modified caseinate were dissolved in distilled water and 1:1 mixed with the matrix. One microliter of the mixture was spotted on the target plate and dried in air for 10 min. The analysis was conducted using a mass spectrometer (Bruker AutoflexII, Germany). The samples were also analyzed using a Coomassie stained 12% SDS−PAGE gel with low molecular weight markers as standards.

Measurement of Zeta Potential. The samples were prepared by dissolving native and modified caseinates (SUCA and EDCA) in 10 mM NaCl solutions at the concentration of 1 mg/mL, and the pH was adjusted using 0.1 M HCl and 0.1 M NaOH. The zeta-potential of 1 mL of each sample was measured using a Zetasizer Nano-ZS instrument (Malvern, UK).

Preparation of O/W Emulsion. Freeze-dried sodium caseinate and modified caseinates were solubilized in 100 mL of Milli-Q water at a controlled pH (mentioned in the figure legends and table) at a protein concentration of 0.3% and 1%. Ninety percent w/w protein solutions were homogenized with 10% w/w flaxseed oil in two steps. A pre-emulsion

was prepared using a stirring-type homogenizer (Heidolph Diax 900, Germany) under constant conditions: 2 times, 2 min at 26000 rpm at room temperature. The main emulsification was performed using a pressure homogenizer (Microfluidics M-110Y, USA) using 100 mL of pre-emulsion at 0 °C and 40 psig (500 bar). After the pre-emulsion was applied, it circulates in the homogenizer for 10 min, during which it passes through the chamber 30 times.

Analysis of Emulsion Properties. pH stability of the emulsions was analyzed by transferring a 2 mL emulsion sample into an eppendorf tube, where after the pH was adjusted from 2 to 12 by adding 1 M NaOH or 1 M HCl solutions. After this, the sample was centrifuged at 6000 rpm for 5 min. The liquid phase was taken by drilling a hole from the bottom of the tube, diluted 100-fold into Milli-Q water, and the absorbance at 633 nm was measured for each sample. To study the storage stability of emulsions, the particle size distribution of freshly made emulsions and emulsions stored at room temperature for 24, 48, and 72 h was measured by laser diffraction (Beckman Coulter LS230, CA). The volume-weighted geometic mean particle diameter (d33) was calculated from the particle size distribution.

RESULTS

Chemical Modification of Sodium Caseinate. Sodium caseinate was succinylated to different extents using succinic anhydride to protein ratios varying from 0.03 g of succinic anhydride per gram of protein to 4 g of succinic anhydride per gram of protein. The relative extent of the chemical modification was measured using the TNBSA assay, which detects the number of free amino groups in a protein and thus can be used to monitor the covalently modified lysine groups as shown in **Figure 1A**. With an increasing dosage of succinic anhydride, the extent of succinylation increased accordingly. Fifteen percent of the lysine groups in sodium caseinate were succinylated when modified by 0.03 g of succinic anhydride per gram of protein, while the extent of modification increased to 94% when the ratio was increased to 0.3 g/g, and with a weight ratio of succinic anhydride to sodium caseinate over 1:1, sodium caseinate was completely succinylated.

The modification of sodium caseinate could also be monitored using SDS−PAGE, and a band shift was detected when sodium caseinate with a different extent of modification was loaded onto the gel. With an increasing amount of succinic anhydride used as modifier, the molecular weight of the products increased, and the caseinate bands shifted upward accordingly (**Figure 1B**, lanes 2−9). From lane 5 to lane 9, the bands have the same relative mobility in the gel, which correlates well with the extent of modification measured using the TNBSA assay. Modification of proteins in general leads to a more heterogeneous population of proteins, which results in a reduced sharpness of the bands in the SDS−PAGE gel and a broadening of the MALDI-TOF MS spectrum (see below).

Succinylation of sodium caseinate was further studied using MALDI-TOF MS by which it was possible to directly measure the extent of modification. It is noteworthy that sodium caseinate is a complex mixture of different casein variants (α, *β*, and *κ* casein), therefore a spectrum that is obtained from native or modified caseinates represents the overall population of unmodified or modified variants rather than homogeneous products. The average molecular mass of the unmodified caseinate mixture was detected to be 23966 Da, and after succinylation to different extents, the mass increased gradually as can be seen in **Figure 2A**. The average

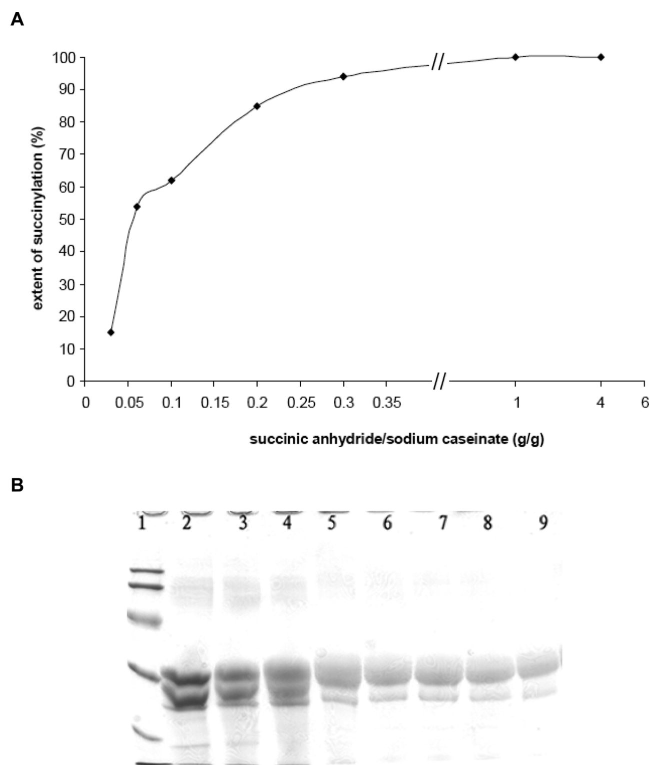


Figure 1. (**A**) Extent of caseinate succinylation measured using the TNBSA method. (**B**) SDS−PAGE analysis of succinylation of sodium caseinate. Lane 1, low molecular weight marker proteins of 97, 67, 45, 30, and 20 kDa; lane 2, sodium caseinate control; lane 3, succinylated caseinate by 0.03 g of succinic anhydride per gram of sodium caseinate; lane 4, succinylated caseinate by 0.06 g of succinic anhydride per gram of sodium caseinate; lane 5, succinylated caseinate by 0.2 g of succinic anhydride per gram of sodium caseinate; lane 6, succinylated caseinate by 0.3 g of succinic anhydride per gram of sodium caseinate; lane 7, succinylated caseinate by 0.6 g of succinic anhydride per gram of sodium caseinate; lane 8, succinylated caseinate by 1 g of succinic anhydride per gram of sodium caseinate (SUCA); lane 9, succinylated caseinate by 4 g of succinic anhydride per gram of sodium caseinate.

number of lysine residues, which had been modified, was calculated by dividing the difference between the molecular mass of modified caseinate and unmodified caeinate obtained from MALDI-TOF MS by the mass of the chemical modifier. The average number of succinylated lysine groups in sodium caseinate, which had been modified with 0.03, 0.06, 0.2 g of succinic anhydride per gram of protein, was 2.5, 5.7, and 11.5, respectively (**Figure 2A**, peaks B, C and D). Interestingly, more and more laser power was required to generate mass spectra from succinylated caseinates with an increasing extent of modification, as can be seen from the reduction of the peak size in **Figure 2A** when succinylation is more extensive. After the succinic anhydride/protein ratio exceeded 0.2 g of succinic anhydride per gram of protein, it was no longer possible to obtain reliable spectra from the modified protein.

EDA modification of a protein converts the carboxyl groups of protein to amino groups. According to Burkey and Gross (*22*), the optimum pH condition for this reaction is at pH 6, while in the case of sodium caseinate, the largest extent of modification was reached at pH 4.7. In our work, partially succinylated caseinate was used as the starting material for the EDA modification because of its improved solubility at the optimum pH of the reaction and more

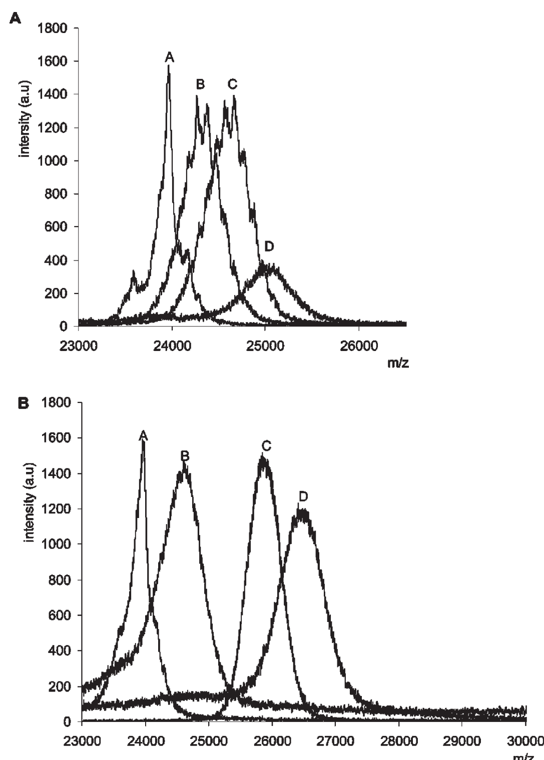


Figure 2. MALDI-TOF mass spectra of modified caseinates. (**A**) Succinylation of sodium caseinate. Spectrum A, sodium caseinate control with molecular weight of 23966 Da; spectrum B, succinylated caseinate by 0.03 g of succinic anhydride per gram of sodium caseinate with molecular weight of 24211 Da; spectrum C, succinylated caseinate by 0.06 g of succinic anhydride per gram of sodium caseinate with molecular weight of 24529 Da; spectrum D, succinylated caseinate by 0.2 g of succinic anhydride per gram of sodium caseinate with molecular weight of 25089 Da. (**B**) EDA modification. Spectrum A, sodium caseinate control with molecular weight of 24148 Da ; spectrum B, succinylated caseinate by 0.06 g of succinic anhydride per gram of sodium caseinate with molecular weight of 24584 Da; spectrum C, EDCA with molecular weight of 25853 Da; spectrum D, EDA modified succinylated caseinate by 1 g of succinic anhydride per gram of sodium caseinate with molecular weight of 26467 Da.

importantly, a much larger extent of modification than that of the modification based on native sodium caseinate. An increase in the amount of free amino groups was observed when the product of the modification reaction was compared with the unmodified sodium caseinate using the TNBSA assay. EDA modified 0.06 g/g succinylated caseinate (EDCA) contained 78% more free amino groups than the native sodium caseinate.

Besides amino acid modification, EDA seemed to have an effect on the mobility of the protein in an SDS−PAGE gel (**Figure 3**, lane 5). The sodium caseinate monomer band had a higher mobility in the gel compared to the succinylated starting material, and treatment with EDC generated cross-linked products, which is due to the formation of intra molecular cross-links. Attempts such as modification using smaller amounts of EDC and lower temperature were made to avoid the cross-linking reaction. With a low dosage of EDC, the cross-linking was effectively suppressed, but meanwhile the pI could not be increased to a desirable level. The modification performed at 4 °C showed no difference from the modification at room temperature (data not shown). For comparison, the EDA modification was applied to other proteins such as BSA and whey protein, which have a more defined globular structure. The modification could be

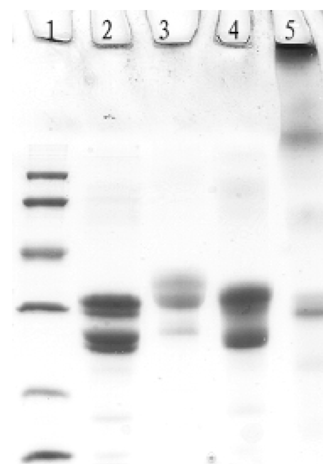


Figure 3. EDA modification. Lane 1, low molecular weight marker proteins of 97, 67, 45, 30, and 20 kDa; lane 2, sodium caseinate control; lane 3, succinylated caseinate by 1 g of succinic anhydride per gram of sodium caseinate (SUCA); lane 4, succinylated caseinate by 0.06 g of succinic anhydride per gram of sodium caseinate; lane 5, EDCA.

performed neatly, showing a large increase in molecular weight in MALDI-TOF MS and a single band with fairly little cross-linked byproduct (data not shown). However, caseinate had been reported as a highly susceptible substrate to the cross-linking reactions. For example, transglutaminase cross-links caseinate very easily by acting on glutamine and lysine residues (*24*); therefore, in terms of EDA modification of caseinate, cross-linking might be an inevitable side reaction.

The increase of molecular mass due to EDA modification was further confirmed by MALDI-TOF MS measurements (**Figure 2B**). As a large portion of the product was cross-linked into modified multimers whose molecular weight was beyond the measurable range, the spectrum observed in MALDI-TOF corresponded to the modified monomer fraction in the mixture. The average number of carboxyl groups that reacted with EDA was calculated to be 17.2 when 0.06 g/g succinylated caseinate was used as the starting material. It was very interesting that after EDA modification, the protein could be measured again in the positive mode using MALDI-TOF MS (**Figure 2B**, peaks C and D). However, since no peak could be obtained from the starting protein, the number of EDA modified residues could not be precisely calculated when fully succinylated caseinate was used as the starting material for the EDA modifications.

Zeta-Potential of Modified Caseinates. The zeta-potential of native sodium caseinate, SUCA, and EDCA was measured and plotted as a function of pH (**Figure 4**). The pH where the zeta potential is zero corresponds with the isoelectric point of the protein, and the more the pH value of a sample differs from the protein's pI, the more charged the surface of the protein is. As can be seen from **Figure 4**, after succinylation to full extent the pI of sodium caseinate decreased from 4.2 to around pH 2.7, and modification of partially succinylated caseinate (0.06 g/g of succinic anhydride to sodium caseinate) with EDA moved the pI of the caseinate mixture up to 9.4. These changes correlate well with the theoretically estimated pI of 4.98 (unmodified), 3.88 (fully succinylated), and 9.54 (EDA modified), respectively. These theoretical values were calculated using the results of the MALDI-TOF MS measurement in combination with an online protein computation tool ProtParam (http://au.expasy.org/tools/protparam.html). The amino acid sequence

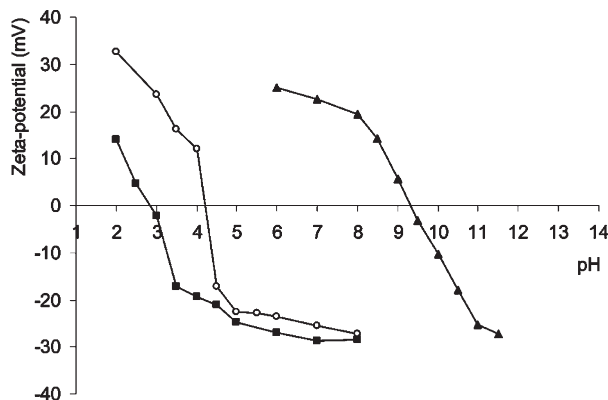


Figure 4. Zeta-potential of native and modified caseinates. Zeta-potential of 1 mg/mL native sodium caseinate (○), SUCA (■), and EDCA (▲) in 10 mM NaCl solutions at different pH values was measured and plotted as a function of pH.

of alfa-S1 caseinate, which is the most abundant caseinate in the mixture, was used as the input parameter of the computational tool.

pH Stability of the Emulsions Made with Modified Caseinates. The emulsifying properties of the modified caseinates with altered isoelectric points were studied at pH values from 2 to 12. The emulsion made of unmodified sodium caseinate collapsed at pH of around 4.5 as can be seen from a visual inspection of **Figure 5A** and from the turbidity measurement shown in **Figure 6** (○); this value correlates well with the pI of sodium caseinate as confirmed by the zeta potential measurement in the previous section. For fully succinylated caseinate (SUCA), with a pI shifted toward a more acidic value, the emulsion lost its stability around pH 2.5−3.5 due to the introduction of additional carboxylate groups in the protein, and as a result, the emulsion was stable at pH values where the emulsion made of unmodified caseinate collapsed as can be seen from **Figure 5C**. The tubidity measurements shown in **Figure 6** (■) confirmed this. EDA modification shifted the pI of caseinate in the opposite direction to 9.4. From a visual inspection of **Figure 5B** and from the turbidity measurement shown in **Figure 6** (▲), the pH at which the emulsion lost its stability was around 8.5−10.5. It can be seen that the emulsion made from this protein mixture containing the EDA modified monomer and multimers had a very satisfactory stability in the pH 3−6 range.

Effect of pH on Emulsification Capability and Emulsion Stability. Besides the emulsions that were prepared at neutral pH 7, the emulsification process was also studied at three different pH values to study the effect of the modifications on emulsification capability and emulsion stability (**Table 1**). At these different pH values, the mean particle diameter was monitored for 72 h. For native sodium caseinate, as pH 5 is close to its isoelectric point (pH 4.2), most of the protein had precipitated and was filtered. The actual concentration of protein that functioned as the emulsifier was very low. Therefore, the emulsion prepared at this pH value was very coarse and unstable. The fresh emulsion already had a particle size larger than 10 μm. At pH values away from its pI, native sodium caseinate had better emulsification capability as the particle size of fresh emulsion was much smaller, and the emulsion was more stable as the mean particle size was still smaller than 1 μm after 72 h. For the modified caseinates, at pH 3 for SUCA and at pH 9 for EDCA, both pH values close to there respective pI, phase separation took place right after the pre-emulsification

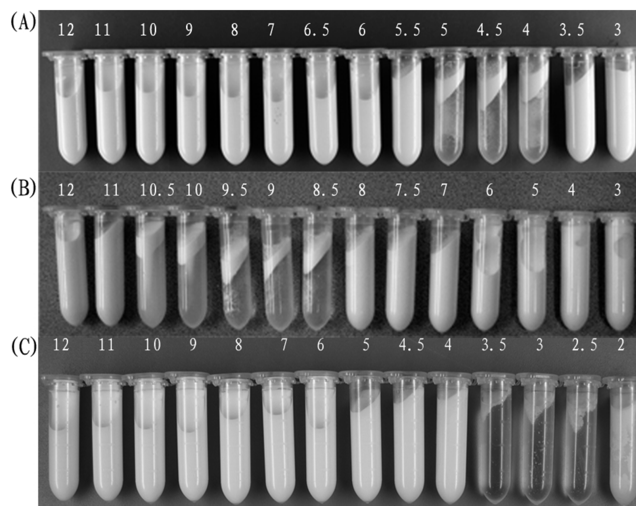


Figure 5. pH stability test of emulsions made of native and modified caseinates (I). Native and modified caseinates were solubilized in Milli-Q water at the concentration of 0.3%, and 90% w/w protein solutions were homogenized with 10% w/w flaxseed oil in two steps as mentioned in Materials and Methods. After adjusting the pH from 2 to12, the samples were centrifuged at 6000 rpm for 5 min. (**A**) Emulsion made of sodium caseinate; (**B**) emulsions made of EDCA; (**C**) emulsion made of SUCA.

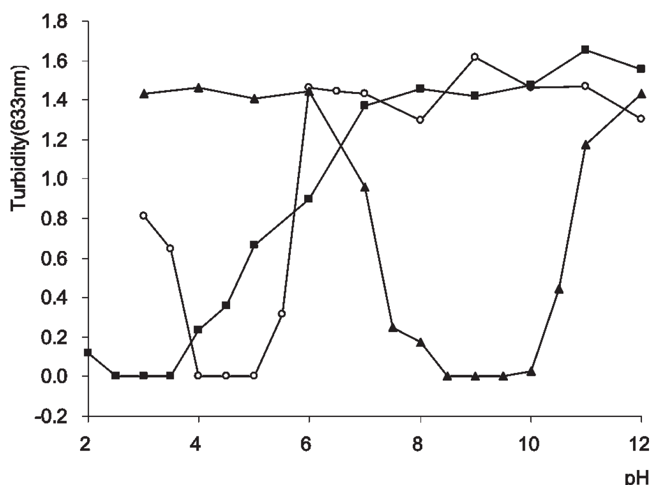


Figure 6. pH stability test of emulsions made of native and modified caseinates (II). The liquid phase of the emulsions made of native caseinate (○), SUCA (■), and EDCA (▲) after centrifugation was taken and diluted 100-fold into Milli-Q water. The absorbance at 633 nm was measured for each sample.

process. Therefore, the sample failed to be further homogenized as the oil phase always floated on top of the mixture and could not be drawn into the pressure homogenizer. At other pH points where emulsification is feasible, good emulsification capability and emulsion stability were observed.

Particle Size Distribution of Freshly Made Emulsions. The particle size distribution of freshly made emulsions using 0.3% (**Figure 7A**) and 1% (**Figure 7B**) of the differently modified caseinates and unmodified caseinate as emulsifiers was measured. Fully succinylated caseinate (SUCA) was found to have the best emulsifying efficiency as it gave the smallest particle size. A minor peak with particle size less than 0.1 μm was observed, but it was assumed to be the protein without oil. This minor peak was also observed with increased protein concentration when native sodium caseinate or EDCA were used. But in the case of SUCA, as the

Table 1. Mean Particle Diameter (μm) of Emulsions Made of the Different Caseins at Different pH Values[a]

	unmodified (μm)				SUCA (μm)				EDCA (μm)			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
pH 3	0.68	0.74	0.86	1.4	ND	ND	ND	ND	0.46	0.70	0.65	0.80
pH 5	10.6	13.0	15.9	34.2	1.4	1.5	2.1	3.2	1.4	1.9	2.2	2.9
pH 7	0.39	0.39	0.41	0.42	0.31	0.31	0.32	0.33	1.23	8.44	9.22	10.0
pH 9	0.26	0.26	0.33	0.49	0.15	0.15	0.16	0.28	ND	ND	ND	ND

[a] ND: Not determined, phase separation took place right after the pre-emulsification process. A protein concentration of 0.3% was used.

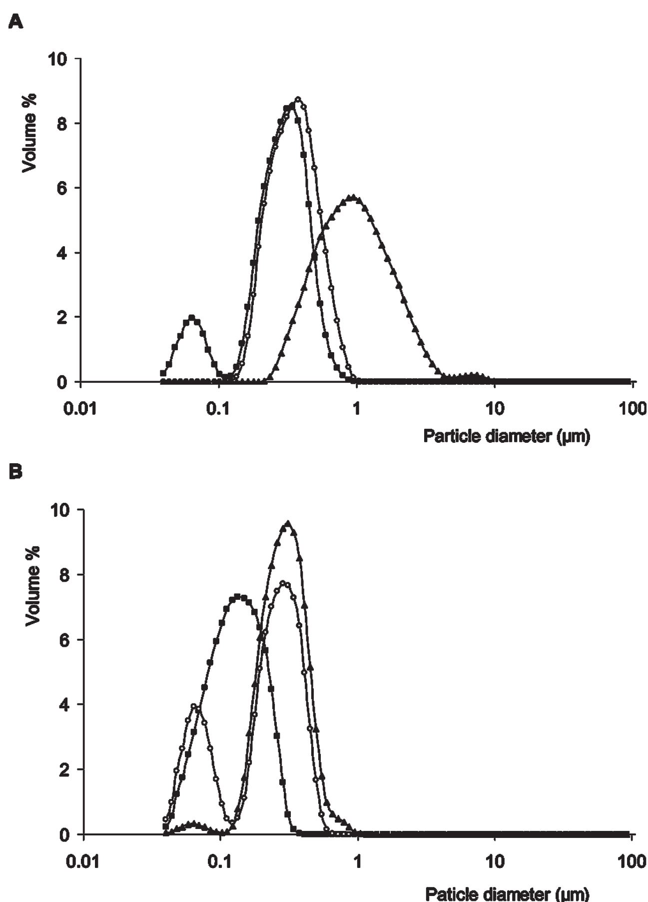


Figure 7. Particle size distribution of freshly made emulsions. The volume distribution curves of freshly made emulsions made of native sodium casseinate (○), SUCA (■), and EDCA (▲) were measured at pH 7 by putting 1−2 drops (~100 μL) of each sample by light scattering using laser diffraction instrumentation. (**A**) Emulsions made of a 0.3% protein solution; (**B**) emulsions made of a 1% protein solution.

amount of excessive protein apparently increased, leading to an increased height of the minor peak and a smaller particle size of the emulsified portion, moving the major peak toward smaller particles size, the two peaks merged into a single one as a result.

Effect of Protein Concentration on Emulsion Stability. The storage stability of the emulsions of modified caseinates was studied by measuring the particle size distribution of the freshly made emulsions compared to emulsions stored at ambient temperature for 24, 48, and 72 h. When the mean particle diameter of emulsions made of 0.3% native caseinate and SUCA (1 g/g succinic anhydride/sodium caseinate) was followed in time, the starting mean particle diameter of fresh emulsions was 0.40 and 0.31 μm, respectively, and no distinct

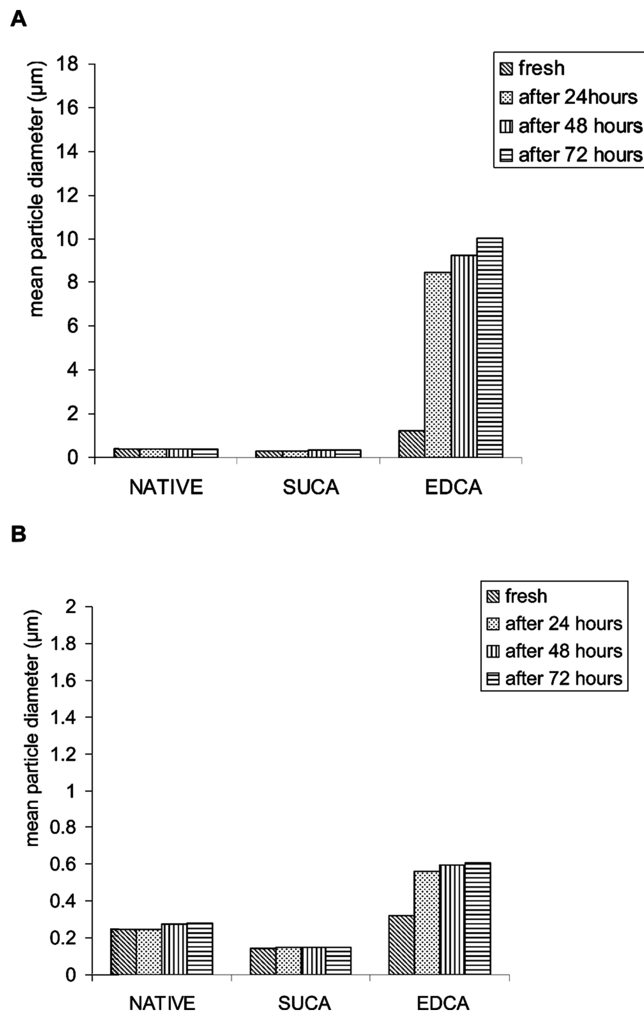


Figure 8. Shelf life of emulsions made of modified caseinates. The particle size distribution of freshly made emulsions at pH 7 and these emulsions stored at room temperature was measured after 24, 48, and 72 h by laser diffraction. The volume-weighted geometric mean particle diameter was calculated from the size distribution and was plotted against time. (**A**) Emulsions made of a 0.3% protein solution; (**B**) emulsions made of a 1% protein solution.

increase in particle size was observed after 72 h. The initial mean particle size of the emulsion made of 0.3% EDCA was 1.23 μm, and within 24 h, a sharp increase in size took place as can be seen from **Figure 8A**. The increase of the protein concentration from 0.3% to 1% shifted the particle size distribution of the fresh emulsions, especially of the emulsions made from EDCA modified caseinate as can be seen in **Figure 8B**. At 1% protein concentration, all particles of the emulsion made from EDCA modified caseinate were smaller than 1 μm, and the storage stability was much better than in the emulsion made at a protein concentration of 0.3%.

DISCUSSION

Succinylation of caseinates was easier to perform compared to the EDA type of protein modification. This reaction took place almost instantaneously when protein and succinic anhydride were mixed, without the need of a catalyst, and no byproducts such as cross-linked caseinates were formed. Another advantage was, as could be shown by MALDI-TOF MS, that the extent of the modification could be subtly controlled using different amounts of the modifier. As expected, the heterogeneity of the protein sample after

modification seemed to be slightly increased as can be judged both qualitatively from the fuzziness of bands in the SDS–PAGE gels and quantitatively from the width of the peaks in the MS spectra. Since the modified protein was used as an emulsifier, this was of less relevance, but it shows the power of mass spectrometry to analyze protein based emulsifiers. The extent of succinylation as a result of different amounts of succinic anhydride used was in agreement with what was reported in the literature: 86% succinylation for beta-casein at 0.2:1 weight ratios of succinic anhydride to casein (*25*). Also, our results support the observation that in order to succinylate caseinate in high levels, large excesses of succinic anhydride are required (*26*, *27*). Interestingly, for the EDA modification, it turned out that the low solubility at the optimum pH for the reaction could be overcome using partially succinylated caseinate as the starting material, having the additional advantage that at same time there are more carboxyl groups available to be modified into amino groups. The use of succinylated caseinate as starting material opens interesting opportunities for further casein modifications.

Zeta potential measurements do not only measure the isoelectric point of modified proteins, but also give an indication of the charge of the modified caseinates at different pH values. A high zeta potential value means that the protein particles are intensively charged on the surface and repel each other. This helps to predict the protein's emulsifying efficiency at a certain pH value, as highly charged caseinate on the oil/water interface can provide emulsion droplets with sufficient repulsive forces against flocculation and coalescence. Therefore, according to the zeta potential measurement, EDCA was expected be a good emulsifier that forms droplets with a highly positive charge at pH values below pH 8. Fully succinylated caseinate, however, is suitable as an emulsifier at pH values above pH 3.5. This prediction based on zeta potential was supported by the results of the pH stability test and the emulsification studies at different pH values. Turbidity above 1.4 was observed in overlapping pH regions for the three differently modified proteins, meaning that the pH range of the protein emulsifier can be modulated by the use of different types of casein modifications.

From pH 2 to 6, EDA modified caseinate has a good pH stability performance, and above pH 6, either succinylated or native caseinate have satisfactory pH stability. When the emulsification efficiency of the different caseinate variants was studied at different pH values, a good correlation with the altered charge behavior of the proteins was also observed. At their isoelectric points, the caseinates could not be solublized and therefore lose their capability to generate and stabilize the emulsion system. Under other pH conditions, a pattern can be found that the further the pH of emulsification is away from emulsifier's pI, the better the achievement of emulsification capability and emulsion stability. This result correlates well with the protein solubility and pH stability pattern observed for the different emulsions.

Full succinylation introduces many carboxyl groups into the protein, resulting in a negative charge when dissolved in distilled water with a pH value around 7. It has been reported that the negatively charged phosphate groups have an unfavorable effect on the ionization process of the MALDI-TOF MS measurement, as the presence of negatively charged carboxyl groups may lead to low ionization efficiency (*28*). This might explain why it was not possible to obtain spectra from highly succinylated casein samples. However, substituting a negatively charged group with a positively charged one has been described as an effective way to improve the MS signal intensity (*29*), and thus, the modification with EDA has been applied to enhance the ionization efficiency in MALDI-TOF MS (*30*). This could explain why the EDA modified samples again showed a sound peak.

The size of emulsion droplets is an important parameter that greatly affects the stability of an emulsion. Measuring the change in the particle size distribution of an emulsion with time can be utilized as a quantitative method of determining emulsifier efficiency and stability. An efficient emulsifier produces emulsions in which the particle size is small ($< 1\ \mu$m) and the distribution does not change over time, whereas a poor emulsifier produces emulsions in which the particle size increases due to coalescence and/or flocculation. Two factors that influence the droplet size are the emulsifier concentration and the speed at which the emulsifier adsorbs in the interface (*3*). In this work, it was apparent that using a higher concentration of protein enabled the protein to cover more interfacial area and generate smaller droplets. At the same caseinate concentration, the particle size of emulsions differed because of the characteristics of each modified caseinate. After succinylation, caseinate was more soluble than the native caseinate at the pH at which the emulsions were prepared. This improved solubility seemed to allow succinylated caseinate to adsorb faster to the interface than the native caseinate. For EDCA, the modified protein was more soluble as well, but it existed in the form of multimers due to the cross-linking reaction. This may have affected the diffusion of EDCA from bulk to interface and made the reorientation process of the protein at the interface slower.

To sum up, succinylation and EDA modification of sodium caseinate resulted in caseinates with altered isoelectric points, and a pH-dependent improvement in solubility and emulsifying properties were achieved. MALDI-TOF MS was shown to be a powerful tool to study these types of modifications. It can very precisely unveil the extent of the modification and help to estimate the pI of the modified emulsifier. An idea about the level of heterogeneity in the products can also be obtained from the peak width. The two different modification methods shifted the pI of caseinate in the acidic or alkaline direction. For proteins, such as sodium caseinate, whose isoelectric point is slightly acidic, the EDA modification is very interesting since it gives the opportunity to study caseinates as emulsifiers in a different pH range. The modified protein had good solubility and emulsifying properties throughout acidic and neutral regions up till pH 7, showing that introducing additional positive charges in caseinate is an efficient way to improve its functionality in this pH range where most of the food applications of emulsion are applied.

The cross-linking reaction that multiplies the molecular mass of caseinate leads to a higher demand on the amount of EDCA to stabilize the emulsion. This side reaction was not observed when the EDA modification was applied to whey protein and BSA. It would be worth studying further the properties of EDA modified whey protein and BSA as emulsifiers as well. EDA itself is not the most ideal compound to produce modified positively charged caseinates for food applications, but this study shows that it would be worthwhile to look for food grade analogues to achieve similar properties. Most interesting would be a protein modification procedure using a biocatalytic approach to reach this goal. Transglutaminase catalyzes the deamination and amine fixation of proteins and has been widely used in the food industry. It would be worth studying whether this type of catalysts or others can be used as tools to modulate protein charge through amino acid modifications.

ABBREVIATIONS USED

EDA, ethylene diamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; MALDI-TOF, matrix assisted laser desorption ionization-time-of-flight; MES, 2-(*N*-morpholino) ethanesulfonic acid; MS, mass spectrometry; O/W emulsion, oil-in-water emulsion; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; pI, isoelectric point; SDS, sodium dodecyl sulfate; TNBSA, 2,4,6-trinitrobenzene sulfonic acid; TFA, trifluoroacetic acid.

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LITERATURE CITED

(1) Zajjc, J. E.; Panchal, C. J. Bio-emulsifiers. *CRC Crit. Rev. Microbiol.* **1976**, *5*, 39–66.
(2) Dickinson, E. Proteins at interfaces and in emulsions. Stability, rheology and interactions. *J. Chem. Soc., Faraday Trans.* **1998**, 1657–1669.
(3) Stauffer, C. Food Emulsifiers. In *Emulsifier: Practical Guides for the Food Indulstry*; Stauffer, C., Ed.; Eagan Press: St. Paul, MN, 1999; pp 39−41.
(4) Damodaran, S. Amino acids, Peptides, and Proteins. In *Food Chemistry*, 3rd ed.; YorkFennema, O. R., Ed.; W.H. Freeman and Co.: New York, 1996; pp 321−430.
(5) Strange, E. D. Chemical properties of thiolated and succinylated caseins. *J. Agric. Food Chem.* **1993**, *41*, 30–36.
(6) Shepherd, R.; Robertson, A.; Ofman, D. Dairy glycoconjugate emulsifiers: Casein-maltodextrins. *Food Hydrocolloids* **2000**, *14*, 281–286.
(7) Howell, N. K. Chemical and Enzymatic Modifications of Food Proteins. In *Food Proteins: Properties and Characterization*; Nakai, S., Modler, H. W., Eds.; VCH Publishers: New York, 1996; Vol.*1*, pp 235−280.
(8) Romoscanu, A. I.; Mezzenga, R. Cross linking and rheological characterization of adsorbed protein layers at the oil-water interface. *Langmuir* **2005**, *21*, 9689–9697.
(9) Morris, G. A.; Sims, I. M.; Robertson, A. J.; Furneaux, R. H. Investigation into the physical and chemical properties of sodium caseinate-maltodextrin glyco-conjugates. *Food Hydrocolloids* **2004**, *18*, 1007–1014.
(10) Oliver, C. M.; Melton, L. D.; Stanley, R. A. Functional properties of caseinate glycoconjugates prepared by controlled heating in the 'dry' state. *J. Sci. Food Agric.* **2006**, *86*, 732–740.
(11) Stevenson, E. M.; Horne, D. S.; Leaver, J. Displacement of native and thiolated β-casein from oil-water interfaces: Effect of heating, ageing and oil phase. *Food Hydrocolloids* **1997**, *11*, 3–6.
(12) Van Hekken, D. L.; Strange, E. D.; Lu, D. P. Functional properties of chemically phosphorylated whole casein. *J. Dairy Sci.* **1996**, *79*, 1942–1949.
(13) Lawal, O. S.; Adebowale, K. O. Effect of acetylation and succinylation on solubility profile, water absorption capacity, oil absorption capacity and emulsifying properties of mucuna bean (*Mucuna pruriens*) protein concentrate. *Nahrung - Food* **2004**, *48*, 129–136.
(14) Achouri, A.; Zhang, W.; Shiying, X. Enzymatic hydrolysis of soy protein isolate and effect of succinylation on the functional properties of resulting protein hydrolysates. *Food Res. Int.* **1999**, *31*, 617–623.
(15) Achouri, A.; Zhang, W. Effect of succinylation on the physicochemical properties of soy protein hydrolysate. *Food Res. Int.* **2001**, *34*, 507–514.
(16) El-Adawy, T. A. Functional properties and nutritional quality of acetylated and succinylated mung bean protein isolate. *Food Chem.* **2000**, *70*, 83–91.
(17) Schwenke, K. D.; Dudek, S.; Knofe, C.; Krause, J. P. Modification of fava bean legumin by limited proteolysis and acylation: Structural and functional aspects. *Recent Res. Dev. Agric. Food Chem.* **2000**, *4*, 217–231.
(18) Bora, P. S. Functional properties of native and succinylated lentil (*Lens culinaris*) globulins. *Food Chem.* **2002**, *77*, 171–176.
(19) Franzen, K. L.; Kinsella, J. E. Functional properties of succinylated and acetylated leaf protein. *J. Agric. Food Chem.* **1976**, *24*, 914–919.
(20) Sundar, R. S.; Rao, D. R. Functional properties of native and acylated peanut proteins prepared by different methods. *Lebensm.-Wiss. Technol.* **1978**, *11*, 188–198.
(21) Kabirullah, M.; Wills, R. B. H. Functional properties of acetylated and succinylated sunflower protein isolate. *J. Food Technol.* **1982**, *17*, 235–249.
(22) Burkey, K. O.; Gross, E. L. Effect of carboxyl group modification on redox properties and electron donation capability of spinach plastocyanin. *Biochemistry* **1981**, *20*, 5495–5499.
(23) Hall, T. C.; McLeester, R. C.; Bliss, F. A. Equal expression of the maternal and paternal alleles for the polypeptide subunits of the major storage protein of the bean *Phaseolus vulgaris* L. *Plant Physiol.* **1977**, *59*, 1122–1124.
(24) Ikura, K.; Kometani, T.; Yoshikawa, M.; Sasaki, R.; Chiba, H. Crosslinking of casein components by transglutaminase. *Agric. Biol. Chem.* **1980**, *44*, 1567–1573.
(25) Hoagland, P. D. Acylated β-caseins. Effect of alkyl group size on calcium ion sensitivity and on aggregation. *Biochemistry* **1968**, *7*, 2542–2546.
(26) Groninger, H. S.Jr. Preparation and properties of succinylated fish myofibrillar protein. *J. Agric. Food Chem.* **1973**, *21* 978–981.
(27) Muhlrad, A.; Corsi, A.; Granata, A. L. Studies on the properties of chemically modified Actin. I. Photooxidation, succinylation, nitration. *Biochim. Biophys. Acta* **1968**, *162*, 435–443..
(28) Janek, K.; Wenschuh, H.; Bienert, M.; Krause, E. Phosphopeptide analysis by positive and negative ion matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1593–1599.
(29) Arrigoni, G.; Resjo, S.; Levander, F.; Nilsson, R.; Degerman, E.; Quadroni, M.; Pinna, L. A.; James, P. Chemical derivatization of phosphoserine and phosphothreonine containing peptides to increase sensitivity for MALDI-based analysis and for selectivity of MS/MS analysis. *Proteomics* **2006**, *6*, 757–766.
(30) Klemm, C.; Schroder, S.; Gluckmann, M.; Beyermann, M.; Krause, E. Derivatization of phosphorylated peptides with S- and N-nucleophiles for enhanced ionization efficiency in matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2697–2705.

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PUBLICATION II

Charge modifications to improve the emulsifying properties of whey protein isolate

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Charge Modifications to Improve the Emulsifying Properties of Whey Protein Isolate

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ABSTRACT: Whey protein isolate was modified by ethylene diamine in order to shift its isoelectric point to an alkaline pH. The extent of the modification was studied using SDS−PAGE and MALDI-TOF mass spectrometry. The modified whey proteins were used as an emulsifier to stabilize oil-in-water emulsions at acidic and neutral pH ranges, and their emulsifying properties were compared with that of the unmodified whey proteins and with the previously studied ethylene diamine modified sodium caseinate. The emulsifying activity of the modified whey proteins was similar to that of the unmodified ones, but the stability of an emulsion at pH 5 was significantly improved after the modification. Charge and coverage of droplet surface and the displacement of the interfacial proteins by surfactant Tween 20 were further studied as a function of pH. As compared with the unmodified whey proteins, the modified ones were proven to cover the interface more efficiently with extensive surface charge at pH 5, although the interfacial layer was less resistant to the surfactant displacement.

KEYWORDS: whey protein isolate, ethylene diamine modification, emulsifying acitivity, emulsion stability

INTRODUCTION

Protein emulsifiers facilitate the formation of oil-in-water (O/W) emulsions and assist the maintenance of emulsion stability (ES). Emulsifying characteristics of proteins are influenced by their adsorption rate and ability to reorganize at the interface. Proteins form visco-elastic interfacial layers, which act as barriers against droplet coalescence, and offer steric and electrostatic repulsions against flocculation.[1,2] Caseins and whey proteins, the two major classes of milk proteins, have been widely used as emulsifiers due to their amphiphilic nature.[3] The emulsifying ability of caseins, especially β-casein, has been extensively studied.[4] Individual caseins have an open flexible structure, whereas whey proteins such as β-lactoglobulin, α-lactalbumin, and bovine serum albumin have more compact globular structures. Because of their flexible structures, caseins can more readily adsorb at the interface as compared to whey proteins, which need longer time to adsorb. However, whey proteins form more viscoelastic interfaces against coalescence.[5] Even if caseins and whey proteins are considered as excellent emulsifiers, their poor emulsifying capabilities at pH values close to their pI (~pH 5) significantly limits their applications in food industries where most of the products are neutral or weakly acidic.[6]

Despite the fact that chemical modifications are usually not favored for food applications due to safety concerns, they have been studied to improve the functionalities of protein emulsifiers. A number of modifications have been reported to improve ES by sterically strengthening the interfacial protein layer.[7] Thicker barriers against flocculation have been obtained by protein−polysaccharide conjugates emulsifiers made by Maillard type reactions.[8] Deamidation has been shown to improve interfacial properties such as layer thickness and structures, providing better emulsion stability against coalescence and heat treatment.[9] Thiolation has been also found to improve the resistance of surface protein layer against displacement by surfactant.[10] As far as electrostatic stabilization is concerned, a handful of modifications have been found to lower the pI of protein emulsifiers and thus provide a pH-dependent improvement of their emulsifying properties. For example, acetylation of proteins eliminates the positive lysyl residues with noncharged side groups. Acylation with succinic or other dicarboxylic anhydrides replaces lysyl residues with negatively charged carboxyl groups. Both of these modifications cause an increase of protein solubility and electrostatic repulsion between droplets at the proteins' pI and thus lead to improved emulsifying activity (EA) and ES.[11]

The use of enzymes as tools to modify protein emulsifiers has gained increasing interest in recent years. Using cross-linking enzymes such as transglutaminase (TGase), milk proteins were polymerized to various extents resulting in stronger cohesive interactions between polymerized β-casein molecules and subsequently in enhanced stability of emulsions.[12] A pH-dependent improvement of EA of cross-linked sodium caseinate by TGase was also reported by Flanagan.[13] Lately, using laccase as the catalyst combined with a layer-by-layer electrostatic deposition technique, Littoz et al. created a cross-linked β-lactoglobulin-beet pectin multilayer to protect droplets against flocculation in the presence of NaCl.[14]

Ma et al. reported a study in which the pH stability of sodium caseinate emulsion was improved by shifting the iso-electric point of casein to the acidic and alkaline sides of the pH range through succinylation and EDA modification.[15] The latter modification converts the negatively charged carboxyl groups into positively charged amino groups, resulting in an increased pI of protein. The EDA modified sodium caseinate had a pI around 9.5 and therefore was able to stabilize the oil−water interface in both the acidic and neutral pH range. However, the sodium caseinate,

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due to its extensive aggregation behavior at pH 5, had to be partially succinylated prior to the EDA modification to maintain good solubility. The majority of the EDA modified sodium caseinate was inevitably cross-linked due to the disordered open structure of individual caseins. As a result of the extensive cross-linking side reaction, the EA of the product was to some extent compromised. The large size of the cross-linked sodium caseinate would lead to a retarded adsorption of the proteins at the O/W interface. Furthermore, due to a high surface concentration of the cross-linked proteins, only a limited surface area could be created and stabilized by insufficient amount of emulsifiers.

The objective of this study was to focus on a protein which can be directly modified by EDA without pretreatment and, more importantly, to produce a nonpolymerized EDA modified protein emulsifier with both improved pH stability and at the same time a satisfactory EA. To minimize the extent of cross-linking side reaction, whey protein isolate (WPI), which has more compact globular structures with limited tendency to aggregate at the isoelectric pH, was chosen as the target protein. In this work, the EDA modification was applied to WPI, and the extent and composition of the modified WPI were investigated. The destabilization process of emulsions made of the unmodified and modified WPI and the surface coverage of these proteins were further studied as a function of pH.

MATERIALS AND METHODS

Materials. Whey Protein Isolate, which was free of lactose (max. 0.5%) and contained a minimum dry protein content of 91%, was obtained from Lacprodan, Arla Foods Ingredients, Viby J, Denmark. Ethylenediamine dihydrochloride was purchased from Sigma (purity ≥99.0%). EDC was purchased from Pierce (purity ≥98.0%). Flaxseed oil was purchased from Elixi Oil Oy (Somero, Finland), where the fatty acid content was the following: 4% 16:0, 3% 18:0, 12% 18:1, 15% 18:2, and 66% 18:3.

Modification of WPI by Ethylene Diamine. The ethylene diamine modification was performed as described by Ma et al.[15] The WPI was dissolved in 0.1 M MES buffer containing 10 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Ethylenediamine dihydrochloride was added at concentration of 20 mM. The pH was adjusted to 5 using a 0.1 M NaOH and 0.1 M HCl solutions. The reaction mixture was stirred for 3 h at room temperature. Dialysis against a 0.02 M NaH_2PO_4 and 0.15 M NaCl solution at pH 7.5 was applied to quench the reaction, and dialysis against sterilized water was applied afterward to remove the unreacted reagents. The EDA modified WPI was recovered by lyophilization. The protein solutions after dialysis were frozen and then lyophilized in a Lyovac GT2 freeze-drier (STERIS Finn-Aqua, Tuusula, Finland) at 0.1 mbar for 3 days to allow a complete removal of water.

Analysis of the Extent of Modification. Matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) was performed to study the extent of the modification. Sinapinic acid was used as the matrix and dissolved to saturation in a 1:1 mixture of 0.1% trifluoroacetic acid (TFA) and acetonitrile. The samples of unmodified WPI and EDA modified WPI were dissolved in distilled water and 1:1 mixed with matrix. One microliter of the mixture was spotted on the target plate and dried in air for 10 min. The analysis was conducted using a mass spectrometer (Bruker AutoflexII, Germany). The samples were also analyzed using a Coomassie stained 18% SDS−PAGE gel with low molecular weight markers as standards.

Emulsion Preparation and Analysis of Emulsifying Properties. The emulsions made of the unmodified and the modified WPIs were prepared at pH 7 as described by Ma et al.[15] Sodium azide (0.02 wt %) was added to the emulsions to prevent microbial growth.

The pH stability and creaming velocity was studied by monitoring the destabilization of emulsions using Turbiscan MA 2000 (Formulaction, Toulouse, France). The pH of emulsions was adjusted to pH 3−10 by adding 1 M NaOH or 1 M HCl solutions. Twenty milliliters of the emulsion at each pH was transferred into a flat-bottomed cylindrical measuring cell and then scanned with a near-infrared light source (λ = 880 nm). The transmission and backscattering profiles were obtained along the height from the bottom to the top of the cell at 1 min intervals for the first 30 min and every 24 h for 1 week. The temperature of measurement was 25 °C. Computation was done in a reference mode where the transmission and backscattering profiles were plotted relative to the data obtained at 0 time. The peak thickness kinetics at the bottom (height 0−10 mm) and top (height 35−45 mm) of each sample was computed at a delta backscattering threshold ((BS min + BS max)/2) as a function of time. The creaming velocity (U) was then calculated from the slope of the plot in the initial stage of creaming (first 30 min): $U = \Delta H/\Delta t$. To further investigate the droplet aggregation at the pI of WPI, the emulsions were also visualized at pH 3, 5, and 7 using confocal laser scanning microscopy (CLSM) as described by Ma et al.[16]

In order to evaluate the EA of WPI and EDA modified WPI at different pH values, another batch of emulsions was prepared by homogenizing 10% flaxseed oil with 90% protein solutions at pH 3, 5, and 7. The particle size distribution of freshly made emulsions and emulsions stored at room temperature for 24, 48, and 72 h was measured by laser diffraction (Beckman Coulter LS230, CA). The measurement was performed in an optical model with fluid refractive index 1.33 and sample refractive index 1.46. The pH of Milli Q water was adjusted to the same value as emulsion samples using 0.1 M HCl and 0.1 M NaOH and used as the measuring media. Samples were consistently taken from the top part of each emulsion. The volume-weighted geometric mean particle diameter (d33) was calculated from the particle size distribution of two batches of fresh emulsions. Two measurements of particle size distribution were conducted from each batch.

Measurement of Zeta-Potential. The zeta potential was measured to evaluate the emulsion stability. The emulsion sample was 1:100 diluted in 2 mL of 10 mM NaCl solution at pH 2−12, and the mobility of oil droplets was measured at 25 °C using a zetasizer (typeNano-ZS, Malvern, Worcestershire, UK). The zeta-potential value was presented as the average of two measurements.

Surface Coverage and Displacement. The above method of homogenization at 500 bar produced emulsions containing small particles that could not be removed afterward by a low protein-binding filter (0.22-μm Millipore) and interfered with the accuracy of the following protein content measurements. Therefore, another batch of emulsions without particles smaller than 0.2 μm was prepared by letting the prehomogenized emulsions at pH 3, 5, and 7 pass through the pressure homogenizer once at 200 bar. The specific surface area of each freshly made emulsion sample was calculated from the particle size distribution measured by laser diffraction (Beckman Coulter LS230, CA). Two milliliters of each emulsion was transferred into an Eppendorf tube and centrifuged at 20 °C and 20000g for 90 min to separate the oil droplets from the aqueous serum phase containing free proteins. The aqueous phase was withdrawn with a syringe and then filtered with a low-protein-binding filter (0.22-μm Millipore). The protein concentration in the serum phase was then determined using the Lowry protein assay.[17] The surface coverage was calculated by dividing the difference between the concentration of the total protein in emulsion and the protein in the serum phase after centrifugation by the known specific surface area.

Surface protein displacement was performed by adding a known amount of water-soluble surfactant Tween 20 into the emulsion aliquots immediately following emulsion formation. Control samples containing various concentrations of Tween 20 without protein were also performed

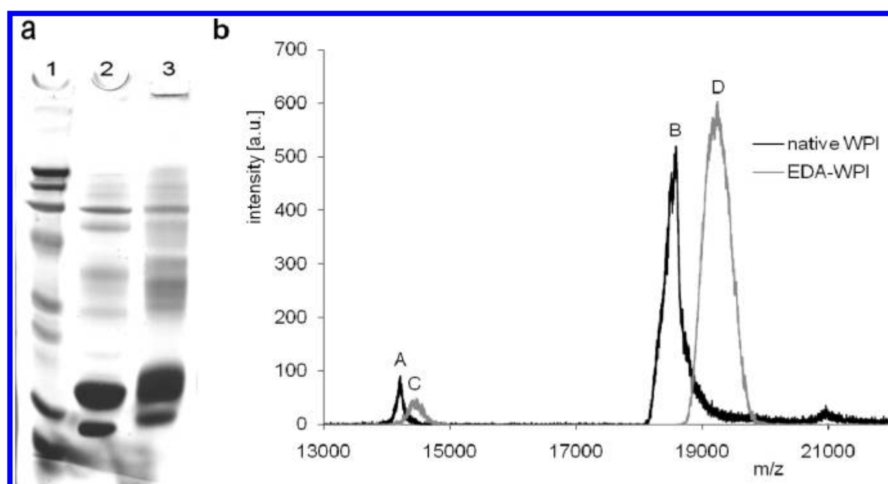

Figure 1. Extent of modification by ethylene diamine. (a) SDS−PAGE analysis of the EDA modified WPI. Lane 1, prestained SDS−PAGE standard proteins of 206.3, 118.1, 97.3, 54.8, 37.7, 29.4, 17.5, and 6.7 kDa (BIO-RAD); lane 2, unmodified WPI control; lane 3, EDA modified WPI (EDA-WPI). (b) MALDI-TOF MS of EDA-WPI. Spectrum A, α-lactalbumin of the unmodified WPI with molecular weight 14229 Da; spectrum B, β-lactoglobulin of the unmodified WPI with molecular weight 18579 Da; spectrum C, α-lactalbumin of the EDA-WPI with molecular weight 14527 Da; spectrum D, β-lactoglobulin of the EDA-WPI with molecular weight 19250 Da.

to confirm that the concentration of Tween 20 used in this experiment did not interfere with the accuracy of protein content measurement. The samples were incubated at room temperature for 1 h to allow competitive adsorption. In the case of the WPI emulsion at pH 5, the sample was vigorously shaken to prevent phase separation during the incubation time. The surface protein concentration after displacement was measured in the same way as that mentioned above for the surface coverage experiment. Displacement of surface protein was plotted against the surfactant-to-protein molar ratio *R*.

RESULTS

EDA Modification of WPI. Modification of a protein using ethylene diamine leads to an increase in the molecular mass for each carboxyl group in WPI that is modified. This mass increase can be monitored qualitatively using SDS−PAGE electrophoresis and quantitatively using MALDI-TOF MS. In SDS−PAGE, two major bands corresponding to β-lactoglobulin and α-lactalbumin in the WPI shifted upward after modification by EDA. Despite the presence of some dimers at a position similar to that of the 37.7 kDa protein marker and a very small amount of extensively cross-linked products in the top of the gel, most of the EDA-WPI existed as monomers (Figure 1a, lane 3). MALDI-TOF MS was used to quantify the occurrence of the EDA modification and to measure the extent of the modification. According to the MALDI-TOF MS analysis, the molecular mass of α-lactalbumin increased from 14208 to 14543 Da and that of β-lactoglobulin from 18579 to 19250 Da (Figure 1b). The exact number of the modified carboxyl groups in WPI could be calculated by dividing the difference between the molecular mass of modified WPI and unmodified WPI by the mass increase as a result of the ethylene diamine modification reaction. As a result, 7.8 of 20 carboxyl groups in α-lactalbumin and 15.6 of 26 carboxyl groups in β-lactoglobulin were modified by ethylene diamine.

Zeta-Potential of the Emulsion Particles. Emulsions stabilized by the unmodified WPI and EDA-WPI were prepared by a two-step homogenization process. Zeta-potential, a reflection of the net surface charge of oil droplets in emulsions was measured based on their electrophoretic mobility and plotted as a function of pH (Figure 2). In the emulsion system, the protein emulsifier forms a charged layer on oil droplet surface and provides a pH-dependent electrostatic repulsive force against aggregation with other droplets. Therefore, the net surface charge of oil droplets is an important parameter for predicting the emulsion stability (ES). High surface potentials ensure a high energy barrier, which causes the repulsion of adjacent emulsion droplets and results in the formation of a stable emulsion. In theory, an emulsion with zeta-potential over $|\pm 30\ \text{mV}|$ is considered as a stable dispersion. Emulsions made of unmodified WPI were found to have good stability when the pH was either below 4.5 or above 6. At pH 5, which is close to the theoretical pI of WPI, the net charge of the protein was nearly zero, and as a result, the WPI emulsions at this pH value were very unstable. With EDA modification, the isoelectric point was shifted from 4.9 to 9.5 as shown by the point where the zeta-potential reached zero. As a result of this, the pH

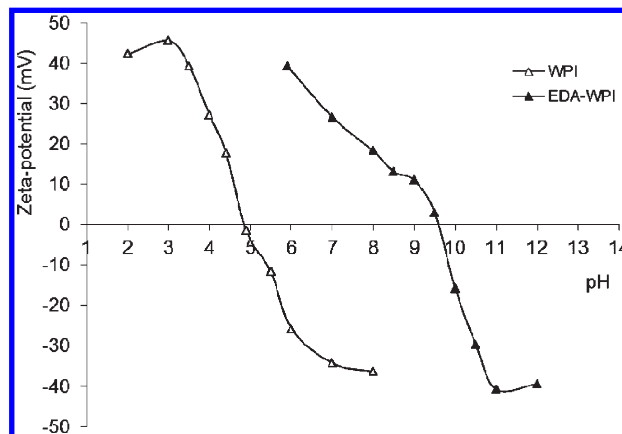

Figure 2. Zeta-potential of oil particles in emulsions made of unmodified and EDA modified WPI. Zeta-potential of unmodified WPI emulsion (△) and EDA-WPI emulsion (▲) 1:100 diluted in 10 mM NaCl solutions at different pH values was measured and plotted as a function of pH. Each point was the average of two replicates, and the standard deviation was less than 10% of the average value.

range where the EDA-WPI failed to make stable emulsions was shifted to the alkaline pH region (pH 8−10), indicating that the modified WPI at the emulsion interface carried enough net charge to provide electrostatic repulsions between droplets and therefore was able to stabilize the emulsions all through the acidic and neutral pH ranges.

pH Stability of the O/W Emulsions. Most of the industrial applications of food emulsions are conducted in acidic or neutral pH environments. The loss of electrostatic repulsion at pI subjects the emulsion to flocculation and possibly, coalescence, which in turn accelerate creaming unless continuous network of flocculated droplets is formed. Therefore, the performance of the modified WPI as an emulsifier at these application pH values was evaluated using backscattering of light to monitor the migration of oil droplets. Turbiscan was used in our study to follow the creaming process in the emulsions. The backscattering intensity is proportional to the amount of particles at different sample height. Therefore, when creaming takes place in an emulsion sample, the backscattering signal decreases at the bottom and increases at the top as the droplets move upward due to the different density of oil and water. For emulsions made of the unmodified WPI, satisfying stability was observed at pH 3 and 7 as the emulsions stayed homogeneous over one week. Only slight creaming took place as seen from the small decrease of the backscattering at the bottom of each sample. At pH 5, backscattering flux, which is influenced by particle size and its volume fraction, was reduced in one week. Increase of particle size decreases the flux in the size range of the oil droplets of the present study. The backscattering flux is also affected by upward migration of the particles, which decreases their volume fraction in the lower parts of the measurement vial. Both creaming and flocculation or coalescence are most likely taking place simultaneously as an increase of the particle size is expected to result in extensive creaming. Transmission was observed at the bottom (height 0−10 mm), and the backscattering at the middle (height 10−35 mm) sharply decreased (Figure 3a, pH 5 line). As a comparison, the emulsions made of EDA-WPI exhibited good stability at all of these three pH values, although creaming at the bottom of the EDA-WPI emulsion seemed to happen slightly faster than in the WPI emulsion at pH 7 (Figure 3b). This slight decrease of stability of EDA-WPI emulsions at pH 7 can be explained by the approach toward the pI of the modified protein.

The migration of emulsion droplets at acidic and neutral pH values was further studied by collecting backscattering data from the first 30 min. The initial rate of creaming (U) was calculated from the top (height 35−45 mm) and the bottom (height 0−10 mm) parts of each emulsion (Figure 4). At pH 3, 4, 6 and 7, the WPI emulsions and EDA-WPI emulsions exhibited similar creaming rates that were small enough (less than 0.03 mm/min) to maintain good emulsion stability. The creaming velocity of the emulsion made of WPI at pH 5 was significantly higher than that of the emulsions at other pH values: 0.08 mm/min at the top and 0.3 mm/min at the bottom. The lower creaming rate at the top than at the bottom may be attributed to a continuous supply of particles from the bottom to the top and the interaction between high concentration of particles which limit the upward movement of each other. The high creaming rate of the WPI emulsion at pH 5 at the bottom caused a quick clarification phenomenon of the sample in a few minutes. The instability caused by droplet aggregation at the pI of WPI was also followed using confocal microscopy (Figure 5). Because of the loss of surface charge of droplets, a great extent of flocculation and coalescence occurred

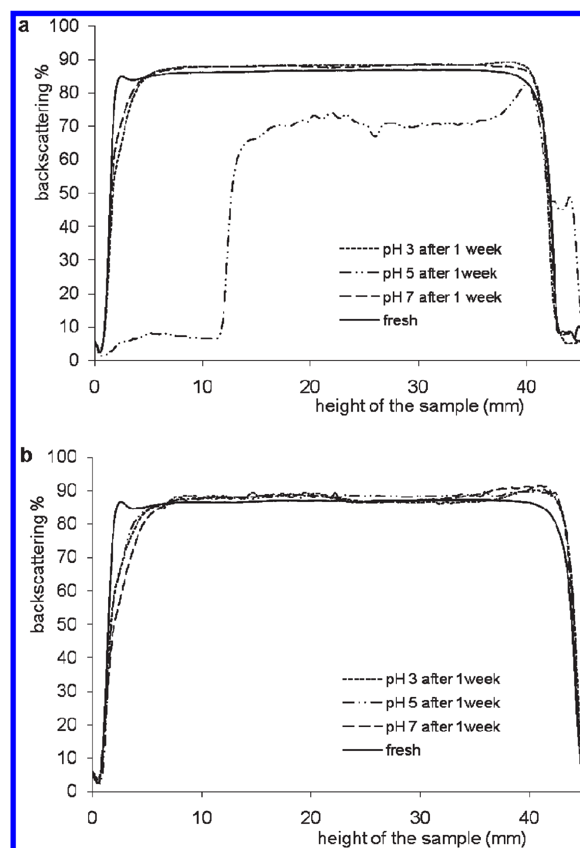

Figure 3. pH stability of emulsions made of unmodified WPI and EDA-WPI. Emulsions made of WPI and EDA-WPI at pH 3, 5, and 7 were stored at RT for 1 week. Backscattering of the fresh and stored emulsions was detected by Turbiscan from bottom to top of a measuring cell and then plotted as a function of sample height. (a) pH stability of emulsions made of unmodified WPI. (b) pH stability of emulsions made of EDA-WPI.

as soon as the pH of the WPI emulsion was adjusted from 7 to 5. An image with higher magnification at the bottom left corner clearly showed the state of the flocculation of small droplets on the surface of a larger one (Figure 5a). Larger particles were formed after 72 h of storage (Figure 5b). As a comparison, the EDA-WPI emulsion was much more stable at pH 5. The droplets with diameters around several hundred nanometers were homogeneously distributed despite the presence of a small number of particles with diameters greater than 1 μm (Figure 5c). Coalescence occurred to a limited extent during the 72 h storage as slightly larger particles were observed (Figure 5d). At pH 3 and 7, the WPI and EDA-WPI emulsions were stable, and the particle size developed in a way similar to that of the EDA-WPI emulsion at pH 5 (data not shown).

Emulsifying Activity and Emulsion Stability. EDA modified caseinate had improved emulsion stability at around pH 5, while a drawback was a decrease of the EA due to the extensive cross-linking side reaction.[15] In this work, the cross-linking of EDA-WPI was shown to occur to a negligible extent (Figure 1a). The EA was studied in the same way as that used previously for EDA modified caseinate. The mean particle size of freshly made emulsions was measured as an indicator of the EA, and the increase of particle size during three-day storage was followed to evaluate the ES (Table 1). The unmodified WPI had good EA at pH values far from its pI. The original particle size was 0.65 μm at

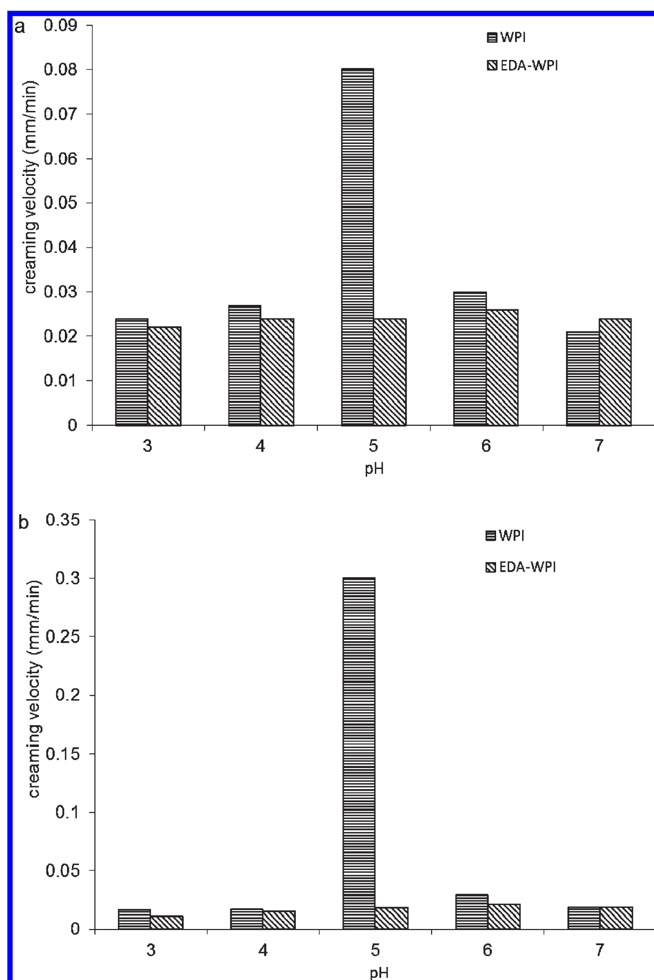

Figure 4. Creaming velocity of emulsions at different pHs. The destabilization of emulsions made of unmodified WPI and EDA-WPI was studied by computing the initial creaming velocity based on the backscattering data that was collected from Turbiscan. (a) Creaming velocity at the top of the emulsions. (b) Creaming velocity at the bottom of the emulsions.

pH 3 and 0.32 μm at pH 7. After 72 h of storage, the particles were still smaller than 1 μm. At pH 5, the unmodified WPI could be still dissolved into the water phase, but the protein aggregated into larger units due to the loss of net charge at its pI.[18] The aggregated state significantly retard the adsorption of the proteins onto the interface, and the electrostatic repulsion between oil droplets was very weak due to the noncharged interfacial proteins. As a result, the unmodified WPI emulsifier made a very coarse emulsion with a mean particle size over 10 μm, and phase separation took place within a few minutes, which made it impossible to further follow the size of larger particles.

The EDA-WPI exhibited better EA and ES over the unmodified WPI at pH 3 and 5. The particle size of the fresh-made EDA-WPI emulsions was much smaller than that of WPI emulsions, and the emulsions maintained excellent stability with insignificant increase of particle size during 3 days of storage at room temperature. The original particle size of the EDA-WPI emulsion at pH 7 was larger than that at pH 3 and 5; however, the size of droplets remained less than 1 μm after 72 h of storage. Compared to extensively cross-linked EDA modified caseinate which has the same pI, but failed to make a stable emulsion at pH 7 (particle

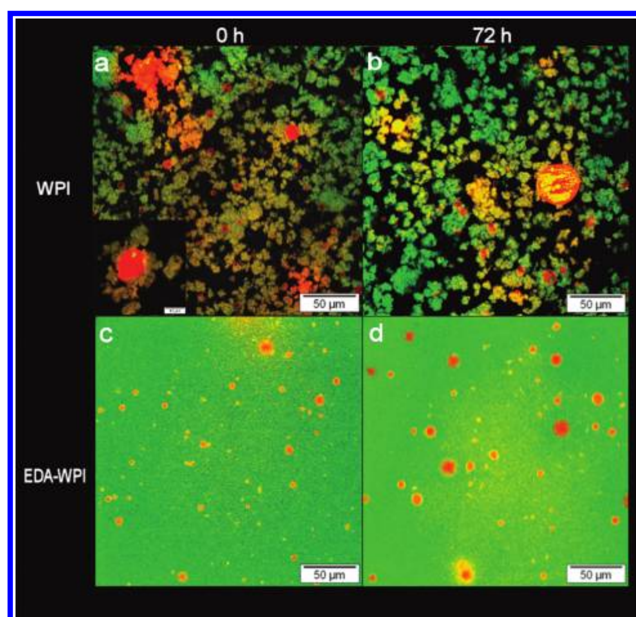

Figure 5. CLSM images of emulsions made of unmodified and EDA modified WPI at pH 5. Size bar = 50 μm. Nile Red was used to stain the oil phase = bright regions. (a) Fresh emulsion made of WPI. (b) Emulsion made of WPI after 72 h storage. (c) Fresh emulsion made of EDA-WPI. (d) Emulsion made of EDA-WPI after 72 h.

size increased from 1.23 to 10.0 μm after 72 h),[15] significant improvements of both EA and ES were observed for EDA-WPI.

Surface Coverage and Displacement of WPI and EDA-WPI. Adsorbed protein layers were further studied by comparing the surface coverage of WPI and EDA-WPI and their resistance to displacement by surfactant Tween 20 as a function of pH. At pH 5, the whey proteins lost their net charge and aggregated into bigger particles. The aggregation could lead to a sharp decrease of the exposed hydrophobic area, which would interact with the surface of oil droplets, and the loss of electrostatic repulsion could promote the binding of more protein from the bulk phase to the interface. As a result, using the same amount of protein for emulsification, significantly higher amount of the unmodified WPI covered unit area of the surface and less surface area was created at pH 5 than at other pH values as shown in Table 2. In contrast, the surface concentration of EDA-WPI was low and did not show significant difference at pH 3, 5 and 7, indicating that EDA-WPI could adsorb more effectively onto the oil surface at both acidic and neutral pH values. A larger amount of protein seemed to be associated with a unit area of interface when the pH got closer to the pI.

The rate and the extent of the protein displacement by the surfactant were observed to be influenced by the surface load of proteins. A higher surface concentration led to a lower degree of displacement. At pH 3 and 7, the extent of displacement of the unmodified WPI was very similar. Around 70% of the surface protein was displaced by Tween 20 when the ratio of surfactant to protein (R) was 10, and at higher surfactant concentrations (R = 40), the surface protein was completely displaced. At pH 5, due to a higher surface concentration of protein which would lead to a denser protective film and less accessibility of surface area caused by the aggregation, only 50% of protein was displaced at a high surfactant concentration (Figure 6a). For EDA-WPI, since this protein was evenly distributed on the droplet surface

Table 1. Volume-Weighted Geometric Mean Particle Diameter d33 (μm) of Emulsions Made of WPI and EDA-WPI at Acidic and Neutral pHs[a]

	unmodified WPI[b]				EDA-WPI[b]			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
pH 3	0.65 ± 0.05	0.63 ± 0.08	0.73 ± 0.03	0.77 ± 0.06	0.39 ± 0.02	0.39 ± 0.04	0.41 ± 0.04	0.43 ± 0.03
pH 5	13.1 ± 1.93	ND	ND	ND	0.48 ± 0.06	0.49 ± 0.03	0.47 ± 0.03	0.50 ± 0.07
pH 7	0.33 ± 0.03	0.42 ± 0.04	0.42 ± 0.03	0.43 ± 0.06	0.78 ± 0.08	0.85 ± 0.06	0.87 ± 0.11	0.93 ± 0.08

[a] ND: Not determined, phase separation took place right after the pre-emulsification process. [b] Mean values ± standard deviation.

Table 2. Surface Coverage (mg/m^2) of WPI and EDA-WPI at the O/W Interface As a Function of pH

	unmodified WPI[a]	EDA-WPI[a]
pH 3	0.25 ± 0.01	0.12 ± 0.01
pH 5	5.04 ± 0.88	0.19 ± 0.02
pH 7	0.12 ± 0.03	0.24 ± 0.01

[a] Mean values ± standard deviation.

without aggregation, the displacement of the surface protein at acidic and neutral pH values seemed to occur in a manner similar to that observed for the unmodified WPI emulsion at pH 3 and 7. At the same concentration of surfactant, the further the pH differed from the pI of EDA-WPI, the more extensively the surface protein was displaced. It was also interesting to notice that the interfacial layer formed by the EDA modified WPI was slightly more resistant to high concentration of surfactant than the unmodified WPI at pH 3 and 7. Results from three replicates consistently showed that around 10% of EDA-WPI remained at the surface when $R = 40$, while the percentage of the displaced unmodified WPI was almost 100% (Figure 6b). This could be explained by the small portion of cross-linked EDA-WPI since cross-linked surface protein was observed in other studies to be more difficult to displace.[19]

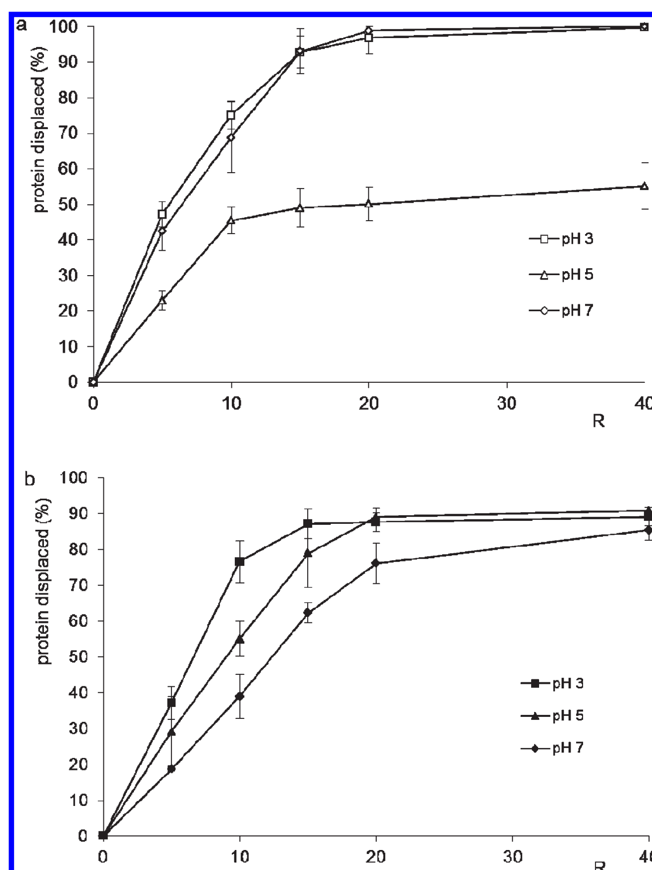

Figure 6. Surface protein displacement. The adsorbed WPI and EDA-WPI on the surface of oil droplets were displaced by surfactant Tween 20 at increasing concentration. The extent of displacement of surface protein was shown by plotting the percentage of displaced surface protein against the surfactant-to-protein molar ratio R. (a) Displacement of WPI at the interface. (b) Displacement of EDA-WPI at the interface.

■ DISCUSSION

In this work, emulsions stabilized by the EDA modified WPI was investigated as a model in which the charge and coverage of O/W interface could be modulated through amino acid modification. Shifting the iso-electric point of protein up to alkaline pH values was shown to be an effective way to enhance ES at acidic and neutral pH range. Since most of the food emulsions are produced in weak acidic or neutral pH range, a protein emulsifier with alkaline pI would form cationic surface layers around the newly created oil droplets and provide strong electrostatic repulsive force against droplet aggregation as long as pH remains neutral or acidic.

The extent of EDA modification, either on caseins which are of open structures or on whey proteins whose structures are globular and more compact, was sufficient to alter the protein's pI up to about 9.5. However, in our previous study the sodium caseinate needed to be partially succinylated prior to the EDA modification in order to overcome its insolubility at the optimal pH of the modification (pH 5), and the EDA modified sodium caseinate was found to be extensively cross-linked during the modification procedure.[15] In this work, WPI was directly modified since the proteins were still soluble at their pI values, and the extent of cross-linking of EDA-WPI was much better controlled under similar reaction conditions. Despite of the partial succinylation, sodium caseinate had a greater tendency to aggregate at pH 5 due to its naturally high surface hydrophobicity, providing more opportunities for a close contact between two caseinate molecules. Furthermore, the open structure of sodium caseinate facilitated the formation of covalent bonds between amino and carboxyl groups, resulting in intra- or intermolecularly cross-linked protein particles. For WPI proteins, which have more compact globular structures and less surface hydrophobicity, self-polymerization would be prevented as the EDC activated carboxyl groups may not locate in accessible positions for another protein molecule due to the steric hindrance and structural distance. As a result, the majority of EDA modified WPI was produced without polymerization.

The EDA modified WPI, without extensive cross-linking, was proved to strengthen the pH stability of emulsions without compromising its EA. A competing cross-linking reaction, depending on the extent, could significantly influence the emulsifying properties of proteins. According to the literature, studies have shown impaired EA and/or ES due to an extensive enzymatic cross-linking treatment; while a small extent of pre-emulsification cross-linking was found to improve the ES against coalescence.[20−22] EDA modified caseinate studied in our previous work was produced as multimers and exhibited good solubility at acidic and neutral pH values. Nevertheless, the EA of the largely cross-linked proteins at neutral pH was reduced most likely due to a retarded adsorption onto droplet surface and an inefficient surface coverage. On the basis of this comparison between EDA-caseinate and EDA-WPI, we draw the conclusion that the studied modification works better with protein emulsifiers that have confined structures. An EDA modified globular protein would exist as a monomer with additional amino groups and has both increased pI and a satisfactory EA.

The adsorption of protein emulsifiers on the oil surface is dependent on the emulsification pH and the conformation of protein. According to the zeta potential result (Figure 2), the proteins had great tendency to aggregate in the pH range around pI $\pm$ 0.5 due to the lack of electrostatic repulsive force. These protein aggregates could adsorb at the O/W interface in a close-packed state with less flexibility to reorganize their structure compared to the monomer proteins. Therefore, an interface formed in this pH region was stabilized by higher concentration of proteins with high surface packing density. Our surface coverage results showed correlation with another study where β-casein was found to form a more condensed film structure with higher surface concentration at its isoelectric point.[23] A dense protein surface layer at the oil−water interface could help to maintain the emulsion quality by providing stronger steric protection against coalescence and retarding the mass transfer of components such as oxygen and pro-oxidants between the aqueous and oil phases. However, the poor surface net charge in this pH range accelerated the aggregation of droplets and eventually led to a quick phase separation. As discussed above, cross-linking of protein emulsifiers to an appropriate extent would be a good approach to strengthen the surface protein film without compromising the surface charge. According to the displacement results, the small portion of cross-linked EDA-WPI with the same surface charge as the uncross-linked one seemed to be more resistant to high concentration of surfactant. This result was in agreement with a previous study in which partially polymerized β-lactoglobulin was found to be more difficult to displace from the oil−water interface.[24,25]

The use of chemicals largely restricts the potential food applications of the EDA modification. However, this chemical method could be considered when pH-dependent stability is needed for nonfood cases. It would be also very interesting to find alternative biocatalytic approaches to increase the isoelectric point of protein molecules, which would open a whole new application area for protein based emulsifiers. In the future, the oxidative stability of emulsions stabilized by EDA modified proteins should be studied since a cationic surface layer has been reported to repel transition metals and other positively charged pro-oxidants away from the oil droplets and thus inhibit the lipid oxidation which often causes quality deterioration in emulsion products.[26]

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ABBREVIATIONS USED

BS, backscattering; EA, emulsifying activity; EDA, ethylene diamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; ES, emulsion stability; MALDI-TOF, matrix assisted laser desorption ionization-time-of-flight; MES, 2-(*N*-morpholino) ethanesulfonic acid; MS, mass spectrometry; O/W emulsion, oil in water emulsion; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; WPI, whey protein isolate; CLSM, confocal laser scanning microscopy

REFERENCES

(1) Dickinson, E. Mixed biopolymers at interfaces: Competitive adsorption and multilayer structures. *Food Hydrocolloids* **2010**, *25*, 1966–1983.

(2) Wilde, P.; Mackie, A.; Husband, F.; Gunning, P.; Morris, V. Proteins and emulsifiers at liquid interfaces. *Adv. Colloid Interface Sci.* **2004**, *108*−*109*, 63–71.

(3) Dickinson, E. Proteins at interfaces and in emulsions. Stability, rheology and interactions. *J. Chem. Soc. Faraday Trans.* **1998**, 1657–1669.

(4) Dickinson, E. Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids Surf., B* **2001**, *20*, 197–210.

(5) Martin, A. H.; Grolle, K.; Bos, M. A.; Cohen Stuart, M. A.; Van Vliet, T. Network forming properties of various proteins adsorbed at the air/water interface in relation to foam stability. *J. Colloid Interface Sci.* **2002**, *254*, 175–183.

(6) Shepherd, R.; Robertson, A.; Ofman, D. Dairy glycoconjugate emulsifiers: Casein-maltodextrins. *Food Hydrocolloids* **2000**, *14*, 281–286.

(7) Chobert, J.-. Milk protein modification to improve functional and biological properties. *Adv. Food Nutr. Res.* **2003**, *47*, 1–71.

(8) Wooster, T. J.; Augustin, M. A. β-Lactoglobulin−dextran Maillard conjugates: Their effect on interfacial thickness and emulsion stability. *J. Colloid Interface Sci.* **2006**, *303*, 564–572.

(9) Day, L.; Xu, M.; Lundin, L.; Wooster, T. J. Interfacial properties of deamidated wheat protein in relation to its ability to stabilise oil-in-water emulsions. *Food Hydrocolloids* **2009**, *23*, 2158–2167.

(10) Stevenson, E. M.; Horne, D. S.; Leaver, J. Displacement of native and thiolated β-casein from oil-water interfaces - Effect of heating, ageing and oil phase. *Food Hydrocolloids* **1997**, *11*, 3–6.

(11) Lawal, O. S.; Adebowale, K. O. Effect of acetylation and succinylation on solubility profile, water absorption capacity, oil absorption capacity and emulsifying properties of mucuna bean (*Mucuna pruriens*) protein concentrate. *Nahrung Food* **2004**, *48*, 129–136.

(12) Liu, M.; Damodaran, S. Effect of transglutaminase-catalyzed polymerization of β-casein on its emulsifying properties. *J. Agric. Food Chem.* **1999**, *47*, 1514–1519.

(13) Flanagan, J.; Gunning, Y.; FitzGerald, R. J. Effect of cross-linking with transglutaminase on the heat stability and some functional characteristics of sodium caseinate. *Food Res. Int.* **2003**, *36*, 267–274.

(14) Littoz, F.; McClements, D. J. Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase. *Food Hydrocolloids* **2008**, *22*, 1203–1211.

(15) Ma, H.; Forssell, P.; Partanen, R.; Seppänen, R.; Buchert, J.; Boer, H. Sodium caseinates with an altered isoelectric point as emulsifiers in oil/water systems. *J. Agric. Food Chem.* **2009**, *57*, 3800–3807.

(16) Ma, H.; Forssell, P.; Partanen, R.; Buchert, J.; Boer, H. Improving laccase catalyzed cross-linking of whey protein isolate and their application as emulsifiers. *J. Agric. Food Chem.* **2011**, *59*, 1406–1414.

(17) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(18) Pelegrine, D. H. G.; Gasparetto, C. A. Whey proteins solubility as function of temperature and pH. *Lebensm.-Wiss. Technol.* **2005**, *38*, 77–80.

(19) Kellerby, S. S.; Yeun, S. G.; McClements, D. J.; Decker, E. A. Lipid oxidation in a menhaden oil-in-water emulsion stabilized by sodium caseinate cross-linked with transglutaminase. *J. Agric. Food Chem.* **2006**, *54*, 10222–10227.

(20) Hiller, B.; Lorenzen, P. C. Functional properties of milk proteins as affected by enzymatic oligomerisation. *Food Res. Int.* **2009**, *42*, 899–908.

(21) Sharma, R.; Zakora, M.; Qvist, K. B. Characteristics of oil-water emulsions stabilised by an industrial α-lactalbumin concentrate, cross-linked before and after emulsification, by a microbial transglutaminase. *Food Chem.* **2002**, *79*, 493–500.

(22) Færgemand, M.; Otte, J.; Qvist, K. B. Emulsifying properties of milk proteins cross-linked with microbial transglutaminase. *Int. Dairy J.* **1998**, *8*, 715–723.

(23) Niño, M. R. R.; Sánchez, C. C.; Patino, J. M. R. Interfacial characteristics of β-casein spread films at the air—water interface. *Colloids Surf., B* **1999**, *12*, 161–173.

(24) Das, K. P.; Kinsella, J. E. Effect of heat denaturation on the adsorption of β-lactoglobulin at the oil/water interface and on coalescence stability of emulsions. *J. Colloid Interface Sci.* **1990**, *139*, 551–560.

(25) Dickinson, E.; Hong, S.-. Surface coverage of β-lactoglobulin at the oil-water interface: Influence of protein heat treatment and various emulsifiers. *J. Agric. Food Chem.* **1994**, *42*, 1602–1606.

(26) Donnelly, J. L.; Decker, E. A.; McClements, D. J. Iron-catalyzed oxidation of Menhaden oil as affected by emulsifiers. *J. Food Sci.* **1998**, *63*, 997–1000.

PUBLICATION III

Improving laccase catalyzed cross-linking of whey protein isolate and their application as emulsifiers

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ARTICLE

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Improving Laccase Catalyzed Cross-Linking of Whey Protein Isolate and Their Application as Emulsifiers

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ABSTRACT: Whey protein isolate (WPI) was chemically modified by vanillic acid in order to enhance its cross-linkability by laccase enzyme. Incorporation of methoxyphenol groups created reactive sites for laccase on the surface of the protein and improved the efficiency of cross-linking. The vanillic acid modified WPI (Van-WPI) was characterized using MALDI-TOF mass spectrometry, and the laccase-catalyzed cross-linking of Van-WPI was studied. Furthermore, the vanillic acid modification was compared with the conventional approach to improve laccase-catalyzed cross-linking by adding free phenolic compounds. A small extent of the vanillic acid modification significantly improved the cross-linkability of the protein and made it possible to avoid color formation in a system that is free of small phenolic compounds. Moreover, the potential application of Van-WPI as emulsifier and the effect of cross-linking on the stability of Van-WPI emulsion were investigated. The post-emulsification cross-linking by laccase was proven to enhance the storage stability of Van-WPI emulsion.

KEYWORDS: Vanillic acid modification, cross-linkability, color formation, emulsion, stability, post-emulsification cross-linking

INTRODUCTION

Whey proteins are widely utilized as food emulsifiers. In an oil-in-water emulsion, whey proteins can readily adsorb onto the surface of newly created oil droplets, forming a protective interfacial layer between the dispersed phase and continuous phase and consequently preventing coalescence as both steric and electrostatic repulsive forces between droplets are provided. Protein-stabilized emulsions have been extensively studied, and various approaches have been investigated in order to improve their stability. Conditions such as pH, temperature, protein concentration, pressure, and addition of polysaccharides during the homogenization have been investigated to establish an optimal protocol for the emulsification process.[1−4] Chemical modifications such as succinylation, acetylation, phosphorylation, thiolation, ethylene diamine modification, as well as Maillard reaction have been used to improve the emulsifying activity and/or emulsion stability of protein emulsifiers.[5−9]

Enzymatic tailoring of proteins by, for example, hydrolysis or cross-linking has also been attempted. Hydrolysis of proteins could influence their emulsifying properties. The effect of some proteolytic enzymes such as pepsin, trypsin, chymotrypsin, and plasmin on dairy protein emulsifiers has been investigated and was found to be very dependent on the selection of enzymes, optimization of the degree of hydrolysis (DH), and other conditions of emulsification.[10−12] Another way to enhance emulsifying properties of protein is cross-linking using enzymes such as peroxidase, tyrosinase, transglutaminase, and laccase.[13−16]

Enzymatic cross-linking can significantly influence the stability of protein-stabilized oil-in-water emulsions by changing the structure of emulsifiers at the interface and/or in the continuous phase. The effect can be, however, dependent on whether the enzymatic reaction is carried out before or after emulsification.[17] Cross-linking of protein emulsifiers before emulsification generally retards the adsorption of protein onto the surface of oil droplets and leads to a lower emulsifying activity.[18] In terms of the emulsion stability, different effects have been reported depending on the enzymes and the extent of cross-linking. Some studies report enhanced emulsion stability (ES) explained by a stronger steric stabilization provided by a thicker layer of protruding branched polymer chain,[18,19] while others observed weakened emulsion stability due to the aggregation of different oil droplets bridged by huge protein polymers.[20,21] In contrast with pre-emulsification cross-linking, post-emulsification cross-linking is believed to be a better approach when aiming at strengthening emulsion stability. Investigations on the stability of emulsions made of α-lactalbumin concentrate after post-emulsification treatment by transglutaminase showed improved ES due to the increase of apparent viscosity in the continuous phase.[17] The same conclusion was drawn from the study of transglutaminase-cross-linked emulsions made of milk proteins.[22] To date, the most commonly used enzyme in the emulsion field is transglutaminase, but some studies on the tyrosinase-catalyzed cross-linking of protein in gels can also be relevant for emulsion makers since similar forces are at stake.[23]

Laccases (EC 1.10.3.2) are blue copper oxidases that can catalyze the oxidation of various aromatic substrates; in particular, different methoxyphenol compounds in, for example, lignin to corresponding phenoxyl radicals. Atmospheric oxygen is used as the electon acceptor by laccase in the oxidation reaction and a byproduct H_2O is formed.[24] The primary radical reaction products can further react nonenzymatically, resulting in a cross-linked product. Laccases have been reported to polymerize various components, such as lignins and other aromatic compounds.[25,26] Although laccases have been reported to be able to oxidize also tyrosine and tyrosine containing peptides,[27,28] the oxidation of

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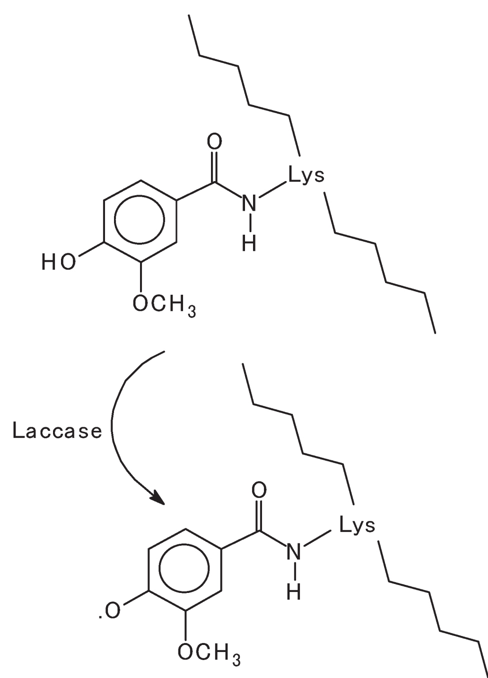

Figure 1. Vanillic acid modified lysine site chain and subsequent activation of the modified protein by lacasse.

proteins has been limited.[22,29] Hence the phenolic groups in protein do not seem to be a very good substrate for laccase-catalyzed reactions. Laccase-catalyzed protein cross-linking has been enhanced by addition of small phenolic compounds as electron transfer mediators which facilitate the formation of homo-cross-links and/or act as bridging agents in heteropolymer structures.[30,31] However, the extent of cross-linking seems to significantly depend on the conformation of the protein substrate. Protein with flexible conformation, casein, for example, can be extensively polymerized by laccase either with high dosage of the enzyme or in the presence of ferulic acid as mediator/bridging agent.[31] On the contrary, the cross-linking of β-lactoglobulin or BSA which has a compact globular structure is only occurring to a very limited extent. Also in this case, the reaction was enhanced by addition of free phenolic acids and high dosage of the enzyme.[29,32] The oxidation and cross-linking in the presence of these commonly used phenolic mediators/bridging agents such as ferulic acid, chlorogenic acid, vanillic acid and caffeic acid cause simultaneous color formation, which is sometimes undesirable for food and other applications.

Introducing methoxyphenol side chains into a protein backbone would be an alternative way to improve the reactivity of protein toward laccase instead of using free phenolic compounds in the reaction. Inspired by lignin, the natural substrate of laccase and abundant with methoxyphenol units, we aimed at creating a "protein−lignin hybrid". This was achieved by chemically incorporating vanillic acid (4-hydroxy-3-methoxybenzoic acid) which is a lignin model compound and commonly used flavoring agent in the food industry with whey protein isolates (WPI) (Figure 1). Making this type of hybrid would give interesting possibilities; one would have different reactive sites in the protein backbone for different biocatalysts to act on. Furthermore, it would give the possibility to form composite biomaterials in which different biopolymers such as, for example, protein and lignin of very different nature are present.

The aim of this work was to covalently functionalize WPI with vanillic acid, whereafter the reactivity of the modified protein toward laccase was studied. Finally, the effect of laccase-catalyzed cross-linking of vanillic acid modified WPI as emulsifier in an oil-in-water emulsion system was studied.

MATERIALS AND METHODS

Materials. Whey protein isolate, which was free of lactose (lactose content below 0.5%) and contained a minimum dry protein content of 91%, was obtained from Lacprodan, Arla Foods Ingredients, Viby J, Denmark. Vanillic acid was purchased from Sigma-Aldrich (purity ≥ 97.0%), and EDC was purchased from Thermo Scientific (purity ≥ 97.0%), Rockford, IL, USA. Sulfo-NHS was purchased from Pierce (purity ≥ 98.5%). Flaxseed oil was purchased from Elixi Oil Oy (Somero, Finland), where the fatty acid content was the following: 4% 16:0, 3% 18:0, 12% 18:1, 15% 18:2, and 66% 18:3. Laccase was produced by *T. hirsuta* and purified by anion exchange chromatography and hydrophobic interaction chromatography.[33] Laccase activity toward ABTS was determined to be 7012 nkat/mL (A protein concentration of 3.9 mg/mL).

Modification of WPI by Vanillic Acid. The modification was performed using a two-step reaction protocol including NHS-ester activation followed by the amine reaction. The NHS-activated vanillic acid was obtained by dissolving vanillic acid, EDC, and sulfo-NHS in DMF at concentrations of 20, 2, and 2.5 mg/mL, respectively, and incubating at room temperature overnight. Then a 10 mg/mL whey protein isolate solution was prepared by dissolving the protein in 50 mM NaP_i buffer pH 7 and 5:1 (volume ratio) mixed with the activated vanillic acid solution. The mixture was stirred at room temperature for 24 h. The product was thoroughly dialyzed against sterilized water and recovered by freeze-drying using a lyophilizer (Steris Lyovac GT 2, Germany). In order to completely remove the residual chemical reagents, the freeze-dried protein was dissolved in sterilized water and purified using an Econo-Pac 10DG desalting column (BIO-RAD). The eluate was collected and freeze-dried again. For the large-scale production of the vanillic acid modified WPI that used as emulsifier, only dialysis was applied.

Analysis of the Extent of Modification. Matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) was performed to study the extent of the modification. Sinapinic acid was used as matrix and dissolved to saturation in a 1:1 mixture of 0.1% TFA and acetonitrile. The samples of unmodified WPI and vanillic acid modified WPI (Van-WPI) were dissolved in distilled water and 1:1 mixed with matrix. One microliter of the mixture was spotted on the target plate and dried in air for 10 min. The analysis was conducted using a mass spectrometer (Bruker AutoflexII, Germany).

Laccase-Catalyzed Cross-Linking. The reactivity of laccase toward the unmodified WPI and Van-WPI was studied by measuring oxygen consumption during the reaction. The enzymatic reactions were monitored with a single-channel oxygen meter (Precision Sensing GmbH, Regensburg, Germany) in a sealed vial (1.84 mL). Unmodified WPI and Van-WPI were dissolved in 5 mM NaP_i buffer pH 6.0 at a concentration of 3 mg/mL. The vial was filled with 1.82 mL of protein solution and capped. Laccase (18 μL) at a dosage of 10 nkat/mg of the modified or unmodified WPI was injected to start the reaction. Consumption of oxygen was monitored as a function of time. Samples containing the unmodified WPI and different concentrations (0.02, 0.2, and 2 mM) of free vanillic acid were also tested with laccase at a dosage of 2.5 nkat/mg of the protein as a control.

The cross-linking of WPI and Van-WPI with 2.5 and 10 nkat/mg laccase dosage as a function of reaction time (0−24 h) was further studied using a 12% SDS gel (BIO-RAD). The cross-linking reaction was stopped by adding 5 μL of SDS-PAGE loading buffer (Tris-HCl with β-mercaptoethanol and SDS) and 5 μL of water into 5 μL of sample and heating at 98 °C for 10 min. Each well in the SDS-PAGE gel was loaded with 15 μL of sample.

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Color Formation. Color formation during the laccase reaction was studied by adding laccase at a dosage of 2.5 nkat/mg into 3 mg/mL WPI, 3 mg/mL WPI with 2 mM free vanillic acid, and 3 mg/mL Van-WPI in 5 mM NaP_i buffer at pH 6.0. The absorbance at wavelength 430 nm was followed using a HITACHI U-2000 spectrophotometer. Reading of the NaP_i buffer was set as a blank.

Preparation of O/W Emulsion and Post-Emulsification Cross-Linking. Freeze-dried WPI and Van-WPI were solubilized in 100 mL of 5 mM NaP_i buffer pH 6.0 at a protein concentration of 0.3%. A two-step homogenization was applied to a mixture of 90% w/w protein solutions with 10% w/w flaxseed oil. A pre-emulsion was prepared using a stirring-type homogenizer (Heidolph Diax 900, Germany) under constant conditions: 2 × 2 min at 26 000 rpm at room temperature. A pressure homogenizer (Microfluidics M-110Y, Newton, MA, USA) was used to perform the main emulsification at 20 °C and 40 psig (500 bar). The pre-emulsion (100 mL) was circulated in the homogenizer for 10 min, during which it passes through the chamber 30 times. After the emulsification, 20 uL of laccase was added to a 20 mL aliquot of the emulsion made at pH 6 (2.5 nkat/mg) and incubated at room temperature for 24 h. Sodium azide (0.02 wt %) was added to the emulsions to stop the laccase reaction and prevent microbial growth. Emulsions without laccase were also prepared as a control. For emulsifying activity study, the emulsions were also prepared at different pH values by adjusting the pH of protein solutions to pH 5, 6, and 7 using 1 M HCl and 1 M NaOH.

Analysis of Emulsion Properties. Particle size distribution of emulsions with and without laccase was measured by laser diffraction (Beckman Coulter LS230, Brea, CA). The measurement was conducted in an optical model with fluid refractive index 1.33 and, sample refractive index 1.46. The pH of Milli Q water was adjusted to the same value as emulsion samples using 0.1 M HCl and 0.1 M NaOH and used as the measuring media. As an indicative parameter of the emulsifying activity of the proteins, the volume-weighted geometric mean particle diameter was determined from the particle size distribution of two batches of fresh emulsions. Two measurements were taken for each batch. For the storage stability study, samples were consistently taken from the top part of each emulsion at 0, 24, and 72 h. Three repeats were performed to confirm the trend of the change of particle size during storage.

The emulsions were also visualized using confocal laser scanning microscopy (CLSM) equipment consisting of a Bio-Rad Radiance Plus confocal scanning system (Bio-Rad, Hemel Hempstead, Hertfordshire, U.K.) attached to a Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan). For imaging, lipids were stained by adding 10 μL of 0.05% (w/v) Nile red in acetone into 1 mL of emulsion. Stained emulsions were examined in 1 mm deep wells on a microscope slide using a 488 nm argon laser line for excitation and a band-pass emission filter at 575−625 nm. Images were assembled of the optical sections taken using a 20× objective (Nikon Plan Apo, numerical aperture 0.75) to the depth of 2 μm with 0.5 μm z step. The stained oil droplets appeared bright. Multiple fields were viewed, and representative images were selected.

RESULTS

Modification of WPI by Vanillic Acid. An NHS-ester activation of vanillic acid followed by a reaction with the lysine amine group was used to modify reactive lysine side chains in WPI. Incorporation of the methoxyphenol groups into the protein through formation of an amide bond leads theoretically to a 150.15 Da mass increase of each reacted lysine residue in WPI. Analysis of the precise molecular mass of unmodified and modified WPI by MALDI-TOF MS was used to confirm the occurrence of the reaction described above and to measure the extent of the modification (Figure 2). Two protein peaks were detected in the mass spectrum of unmodified WPI corresponding to α-lactalbumin (M_w 14118 Da) and β-lactoglobulin (M_w 18481 Da).

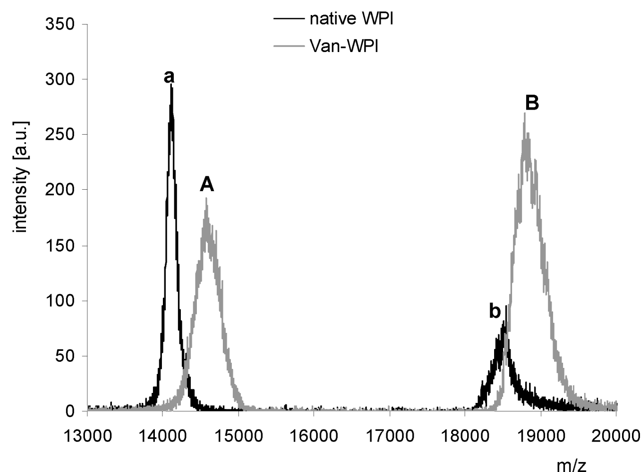

Figure 2. MALDI-TOF mass spectra of the modified WPI. (a) Unmodified α-lactalbumin with molecular mass 14 118 Da; (b) unmodified β-lactoglobulin with molecular mass 18 481 Da; (A) vanillic acid modified α-lactalbumin with molecular mass 14 557 Da; (B) vanillic acid modified β-lactoglobulin with molecular mass 18 798 Da.

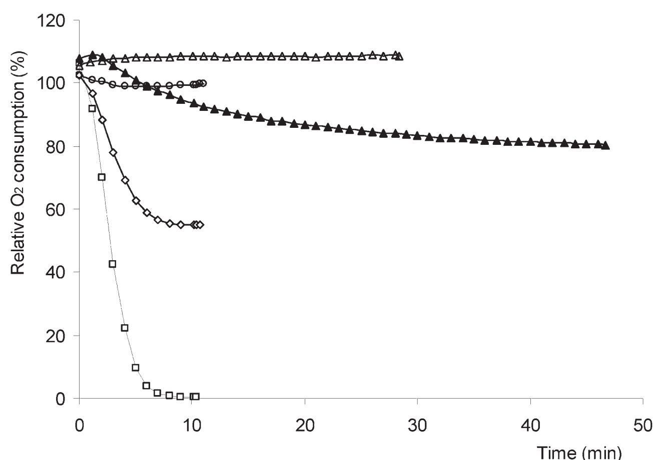

Figure 3. Oxygen consumption measurement of laccase reaction of unmodified WPI with free vanillic acid and modified WPI. 2.5 nkat/mg laccase was added into WPI with 2 mM (□), 0.2 mM (◇), and 0.02 mM (○) free vanillic acid and 10 nkat/mg laccase was added into WPI alone (△) and Van-WPI (▲).

The modified WPI (Van-WPI) had two similar peaks, but with higher molecular masses, that is, 14 557 and 18 798 Da as compared to the unmodified WPI. From the spectra, it can be seen that α-lactalbumin seemed to be more prone to the vanillic acid modification since a larger increase in molecular mass was detected compared to the modified β-lactoglobulin. The average number of lysine residues that were modified was calculated by dividing the difference between the molecular mass of WPI and Van-WPI by the mass of vanillic acid. According to this calculation, on average 2.9 of 12 lysine residues in α-lactalbumin and 2.1 of 16 lysine residues in β-lactoglobulin were modified by vanillic acid.

Laccase-Catalyzed Cross-Linking of Vanillic Acid Modified WPI. Vanillic acid modified WPI contains methoxyphenol side groups that are reactive toward laccase and therefore is expected to have an improved cross-linkability compared to the unmodified WPI. The reactivity of Van-WPI with laccases was studied using oxygen consumption measurement and SDS-PAGE electrophoreses.

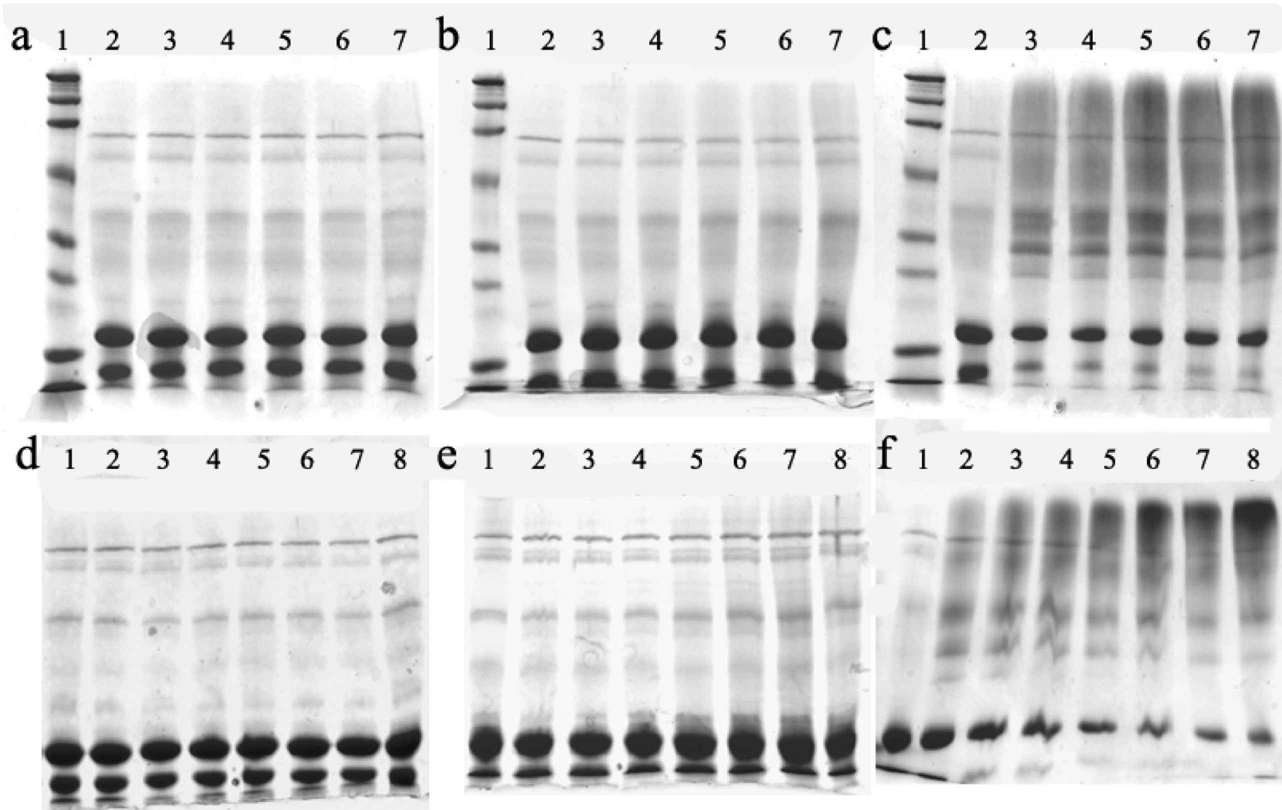

Figure 4. SDS-PAGE of the unmodified and modified WPI after laccase reaction. The cross-linking of WPI and vanillic acid modified WPI (Van-WPI) was performed at a laccase dosage of 2.5 nkat/mg (a−c) and 10 nkat/mg (d−f). (a) WPI with 2.5 nkat/mg laccase; (b) WPI with 2 mM free vanillic acid and 2.5 nkat/mg laccase; (c) Van-WPI with 2.5 nkat/mg laccase. (a−c) lane 1, prestained SDS-PAGE standard proteins of 206.3, 118.1, 97.3, 54.8, 37.7, 29.4, 17.5, and 6.7 kDa (BIO-RAD); lane 2, cross-linked sample at 0 h; lane 3, cross-linked sample at 2 h; lane 4, cross-linked sample at 4 h; lane 5, cross-linked sample at 8 h; lane 6, cross-linked sample at 12 h; lane 7, cross-linked sample at 24 h. (d) WPI with 10 nkat/mg laccase; (e) WPI with 2 mM free vanillic acid and 10 nkat/mg laccase; (f) Van-WPI with 10 nkat/mg laccase. (d−f) lane 1, cross-linked sample at 0 h; lane 2, cross-linked sample at 10 min; lane 3, cross-linked sample at 20 min; lane 4, cross-linked sample at 30 min; lane 5, cross-linked sample at 1 h; lane 6, cross-linked sample at 2 h; lane 7, cross-linked sample at 4 h; lane 8, cross-linked sample at 12 h.

The conventional approach to improve cross-linking by adding free phenolic compound to the unmodified protein was used as a comparison.

Oxygen consumption measurements were performed to study the radical formation stage of the laccase-catalyzed cross-linking reaction (Figure 3). For the samples containing unmodified WPI and free vanillic acid, an increasing amount of oxygen was consumed depending on the concentration of vanillic acid: in the presence of 2 mM vanillic acid, all the oxygen in the reaction vial was consumed in 10 min, 47% of oxygen was consumed in the sample with 0.2 mM vanillic acid, and for a mixture with 0.02 mM vanillic acid only 3% oxygen was consumed in the same time. The unmodified WPI solution without addition of free vanillic acid did not show consumption of oxygen although the reaction was carried out with a higher laccase dosage of 10 nkat/mg for a longer time. The oxidation of Van-WPI was limited when 2.5 nkat/mg laccase was used (data not shown). When a higher dosage of 10 nkat/mg laccase was used, about 29% of the oxygen in the vial was consumed within 50 min.

The extent of laccase-catalyzed cross-linking of Van-WPI was studied using SDS-PAGE electrophoresis (Figure 4). Unmodified WPI, a mixture of WPI and free vanillic acid, and Van-WPI were treated with different dosages of laccase and monitored for different times. The efficiency of cross-linking of unmodified WPI was very low: a little increase of intensity of dimers of the unmodified α-lactalbumin and β-lactoglobulin at molecular mass around 29.4 kDa and 37.7 kDa was observed, indicating a slight cross-linking of the unmodified WPI by laccase after 24 h (lane 7, panel a). Addition of free vanillic acid improved the cross-linking to a small extent as slightly more dimers of α-lactalbumin and β-lactoglobulin were formed (lanes 4−7, panel b). However, most of the unmodified WPI stayed non-cross-linked, and increasing the enzyme dosage barely improved the cross-linking (panels d and e). In contrast, at an enzyme dosage of 2.5 nkat/mg, the majority of vanillic acid modified α-lactalbumin was cross-linked after 2 h as can be seen from lane 3 in panel c. Vanillic acid modified β-lactoglobulin took a longer time to be cross-linked. A decrease of intensity of the band of vanillic acid modified β-lactoglobulin was observed, although uncross-linked monomers still could be detected after 24 h. The molecular mass of the cross-linked proteins increased with duration of the reaction, ranging from 29 kDa to over 206 kDa as was detected in the top of each lane (panel c). At a higher dosage of laccase, the cross-linking of the Van-WPI was greatly accelerated (panel f). After 30 min, almost all the vanillic acid modified α-lactalbumin and nearly half of the modified β-lactoglobulin was already heavily cross-linked. With a longer reaction time, after 4 h most of the Van-WPI was extensively cross-linked, assembling at the top of the lane (lane 7, panel f).

The SDS-PAGE gel and oxygen consumption results suggested that the method to improve the cross-linking by adding free

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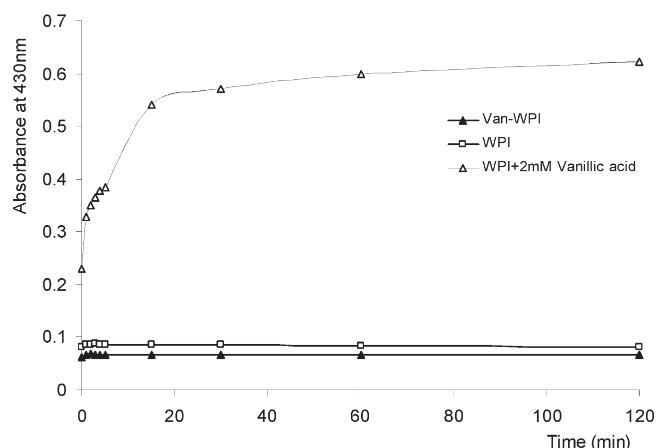

Figure 5. Color formation during the laccase reaction. 2.5 nkat/mg laccase was added into WPI (□), a mixture containing WPI and 2 mM free vanillic acid (△), and Van-WPI(▲). The color formation was followed by plotting the absorbance at wavelength 430 nm against time.

vanillic acid made a quick oxidation of the free phenolic compounds by laccase, while the nonenzymatic reaction during which the free radicals mediated the cross-linking of the protein was much delayed as it took a long time for the free radicals to reach the tyrosine residues inside the protein structure, and the radicals formed in unmodified WPI may not be able to react with radicals in other molecules due to steric hindrance. The major reaction occurred in this "vanillic acid mediated" system was the oxidation and self-polymerization of vanillic acid, which proved by the change in color (Figure 5). In comparison, the polymerization of Van-WPI was proved to occur simultaneously with the radical formation as a small amount of the Van-WPI was already cross-linked during the first 10 min, and by the end of the radical formation stage the Van-WPI was extensively polymerized (Figures 3 and 4).

Color Formation during the Cross-Linking Reaction. The color formation during the laccase reactions with unmodified and modified WPI was studied, and the results are shown in Figure 5. A mixture of 3 mg/mL unmodified WPI and 2 mM vanillic acid had a very slight orange color when free vanillic acid was dissolved, while the other two samples without the free vanillic acid stayed colorless. Treated with laccase, a strong orange color was formed within 15 min in the mixture of WPI and vanillic acid which could be monitored as a strong absorbance increase at wavelength of 430 nm. No color formation was observed in WPI or Van-WPI after laccase treatment, and the absorbance remained below 0.1 within 2 h.

Effect of the Cross-Linking on the Emulsifying Properties of Van-WPI. The volume-weighted geometric mean particle diameter of freshly made emulsions was determined as an indication of emulsifying activity of WPI and Van-WPI. For both the unmodified WPI and Van-WPI, better emulsifying activity was found with increasing pH (Table 1). The emulsifying activity of the unmodified WPI was slightly better than that of modified WPI at pH 6 and pH 7, resulting in a smaller mean particle size. At pH 5, the unmodified WPI could not be emulsified as the oil and water phases separated immediately after the stirring type homogenization. The vanillic acid modified WPI showed an improved emulsifying activity over the unmodified WPI at pH 5. In theory, the vanillic acid modification changes the isoelectric point (pI) of WPI, since positively charged

Table 1. Emulsifying Activity of WPI and Van-WPI at Different pH Values by Measuring the Mean Particle Diameter from Volume Distribution[a]

	pH 5	pH 6	pH 7
WPI (μm)	ND	0.84 ± 0.07	0.72 ± 0.13
Van-WPI (μm)	3.1 ± 0.4	1.0 ± 0.0	0.93 ± 0.12

[a] ND: Not determined; phase separation took place right after the pre-emulsification process.

lysine residues are converted into neutral side groups. The pI of Van-WPI was not significantly changed; the pI of the vanillic acid modified α-lactalbumin was shifted from 4.9 to 4.6 and from 4.9 to 4.8 for β-lactoglobulin according to the method of pI calculation described by Ma et al.[8] This small decrease of pI could explain the slightly improved emulsifying activity of Van-WPI at pH 5.

The effect of post-emulsification cross-linking on the stability of emulsions was studied by comparing the particle size distribution before and after the laccase treatment. Since addition of free vanillic acid caused collapse of emulsions (data not shown) and it did not make significant improvement of cross-linking (Figure 4), the unmodified WPI emulsion was prepared without vanillic acid and compared with the Van-WPI emulsion for the emulsion stability study.

The laccase-catalyzed post-emulsification cross-linking was performed on emulsions made of WPI and Van-WPI at pH 6, and an improved shelf life was observed for the cross-linked emulsion of Van-WPI (Figure 6). For the emulsion made of WPI, 62.8% of the particles had a diameter of less than 1 μm, and the presence of relatively larger particles from 1 to 28.7 μm was also detected. Laccase treatment for 24 h did not improve emulsion stability, and probably due to the addition of enzyme the laccase treated emulsion of WPI was shown to be slightly less stable than the one without enzyme treatment. The volume-weighted geometric mean particle diameter of a fresh WPI emulsion was 0.84 μm, and after 72 h the particle size significantly increased to 1.54 μm for the emulsion without laccase and 2.11 μm for the laccase treated one (panels a and b, Figure 6). The fresh emulsion made of Van-WPI contained 43.2% of particles less than 1 μm and the larger particles ranged from 1 to 4.4 μm. The average particle size of the fresh emulsion was determined to be 1.02 μm. Without treatment with laccase, the average particle size of the Van-WPI emulsion increased to 1.37 μm and a small peak was observed from the particle distribution curve, representing the generation of large particles sized from 10 to 37.9 μm during storage. The laccase treated emulsion of Van-WPI exhibited the best stability among all the samples. The mean particle diameter increased from 1.02 to 1.05 μm after 72 h. Despite the fact that a small amount (0.8%) of particles that were less than 1 μm had become slightly larger during the storage, the overall particle size stayed within the range from 0.1 to 4.8 μm, and no larger particles were observed (panels c and d, Figure 6).

The increased particle size suggested the aggregation of oil droplets in the non-cross-linked emulsions. Confocal microscopy was used to get a direct vision of droplet behavior. For fresh emulsions made of both unmodified and modified WPI, the oil droplets were homogeneously distributed and the diameter of most of the oil droplets was less than 1 μm despite the presence of a small number of bigger droplets (panels a and d, Figure 7). Coalescence was found to occur during the storage of emulsions without cross-linking as large oil droplets were detected after 72 h

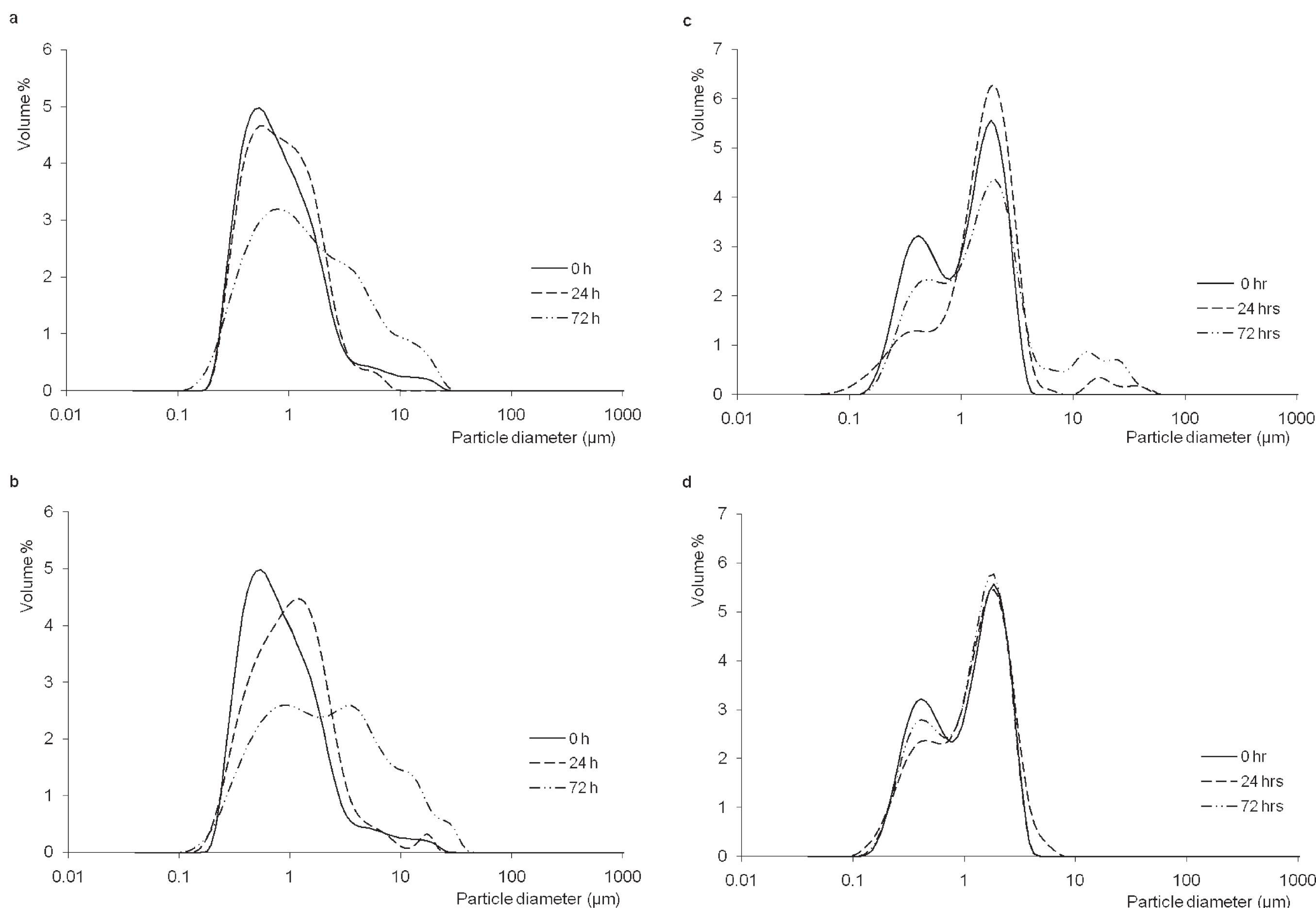

Figure 6. Effect of cross-linking on particle size distribution of emulsions. The particle size distribution of freshly made emulsions and emulsions stored at room temperature was measured after 24 and 72 h storage. The volume-weighted geometic mean particle diameter (d33) was calculated. (a) Emulsion made of the unmodified WPI without laccase treatment; (b) laccase-treated emulsion made of the unmodified WPI; (c) emulsion made of the Van-WPI without laccase treatment; (d) laccase-treated emulsion made of the Van-WPI. The experiment was repeated three times. The standard deviation of the particle diameter of the fresh emulsions was less than 10% of the mean value and around 20% for the aged emulsions. However, the trend of size change was consistent.

(panels b, c, and e, Figure 7). Due to stronger attractive interaction, the droplet concentration around these large particles was higher than other regions with smaller droplets. For the laccase-treated Van-WPI emulsion, the droplets stayed almost the same size as in the fresh sample, indicating better storage stability against coalescence. The emulsion droplets were homogeneously distributed, and no concentration effect indicating flocculation as observed for the non-cross-linked samples was found (panel f, Figure 7). The increased particle size in the non-cross-linked emulsions significantly accelerated the creaming of droplets. As a result of this, clarification was visually observed at the bottom of these samples and the large oil droplets on the top eventually merged together into a separate oil layer with light yellow color (Figure 8).

DISCUSSION

Laccase Catalyzed Cross-Link Formation. The exact mechanism of laccase-catalyzed cross-linking of proteins is not completely understood so far. The only known mechanism is that cross-linking of tyrosine-containing proteins or peptides is primarily based on formation of isodityrosine bonds, with a small amount of dityrosine and disulfide bonds. Isodityrosine bonds are formed between hydroxyl and tyrosyl radicals that are located in different protein molecules; therefore, the accessibility of tyrosine residues in protein is expected to be very important for its susceptibility to a laccase-catalyzed reaction.[27,34] Actually, tyrosine alone or in short peptides can be directly oxidized by laccase with subsequent formation of cross-links,[27] while in proteins that have a compact structure internal tyrosine residues may not be directly accessible for the enzyme. Phenolic acids, being small molecules that can easily interact with both the enzyme and the protein substrates, are able to enhance the cross-linking either by transferring electrons to protein which can be cross-linked via radical reactions or by directly bridging the connection between two tyrosine residues from protein.

The modified WPI could be efficiently cross-linked by laccase, whereas when free vanillic acid was used with the unmodified WPI; the main reaction seemed to be polymerization of vanillic acid by itself. The vanillic acid mediated the cross-linking reaction by transferring electrons and creating reactive radicals in the protein. It is also possible that some vanillic acid was incorporated into the cross-linked protein. The role of phenolic acid as a bridging agent in cross-linking had been proved by Mattinen et al.[27] Another study on laccase-catalyzed cross-linking of β-lactoglobulin with ferulic acid speculated the incorporation of ferulic acid in the cross-linked structure.[29] The introduction of

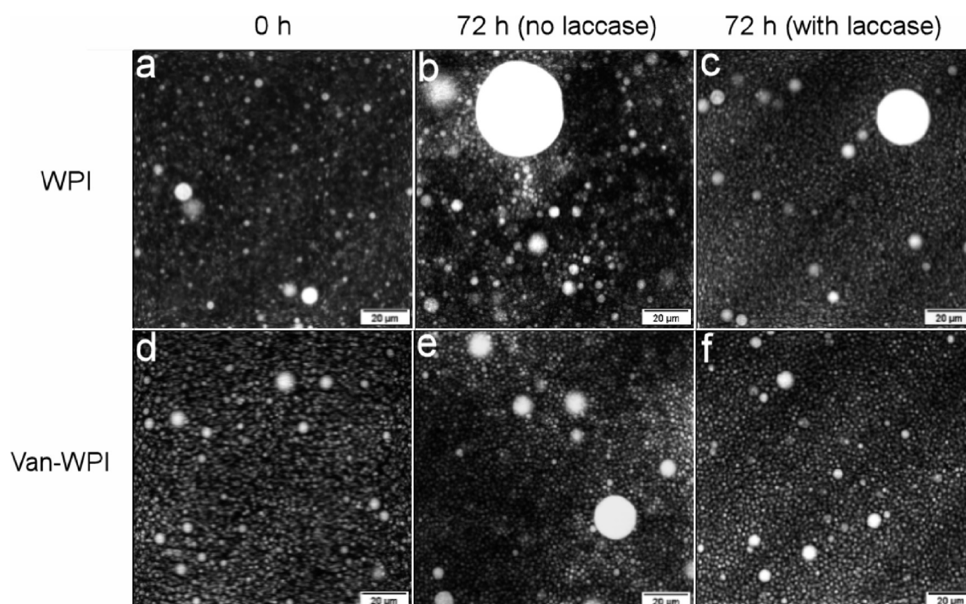

Figure 7. CLSM images of emulsions made of the unmodified and modified WPI with or without laccase treatment. Size bar = 20 μm. Nile Red was used to stain the oil phase = bright regions. (a−c) Different emulsions made from WPI; (d−f) different emulsions made from Van-WPI.

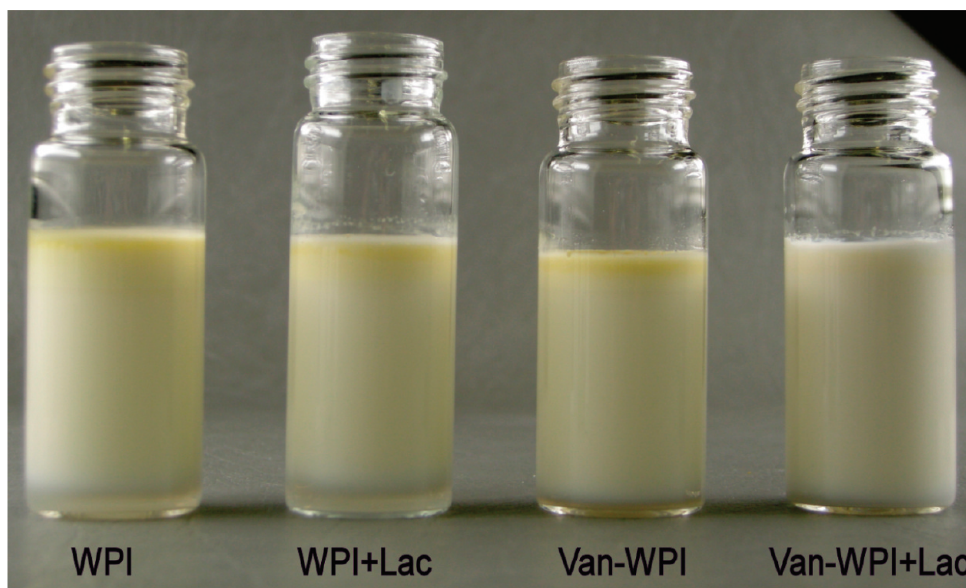

Figure 8. Visual observation of aged emulsions. The emulsion samples were stored at room temperature for 1 week. Clarification and creaming were observed, respectively, from the bottom and the top of tubes.

methoxyphenol groups onto the protein surface enhanced the laccase-catalyzed cross-linking reaction. These introduced methoxyphenol groups on the protein surface seem therefore to be accessible for laccase. Using this system, it became possible to perform the cross-linking reaction in a "phenolic-compound-free" system where the laccase enzyme seems to work directly on the protein; both the enzymatic oxidation reaction indicative for radical formation and the subsequent nonenzymatic cross-linking could be monitored.

The interaction of laccase with vanillic acid modified WPI is also interesting from a laccase substrate binding pocket architecture perspective and its natural ability to oxidize polymeric phenol containing substates. Recently determined complex structures with a methoxy phenol compound bound in the substrate binding pocket and modeling studies show that the active site in laccases is a relatively hydrophobic cavity whose size and shape seem to vary, when laccases of different origins are compared.[35,36] From these studies, it can be seen that the para-position of the benzene ring relative to the phenolic hydroxyl group seems to be facing toward the solution. The orientation makes interactions with methoxyphenol groups incorporated in a polymeric structure such as lignin feasible. The interaction of laccase with an artificial methoxyphenol containing polymer observed in this study supports these structural observations.

The two major components of WPI, α-lactalbumin and β-lactoglobulin, have different reactivity toward the laccase

enzyme. α-Lactalbumin has been reported to be relatively more susceptible to laccase although the extent of cross-linking is still very limited.[32] The vanillic acid modification improved the reactivity of both α-lactalbumin and β-lactoglobulin. The vanillic acid modified α-lactalbumin was the preferred substrate and thoroughly cross-linked by a low dosage of laccase, while a higher dose of the enzyme was needed for extensive cross-linking of the vanillic acid modified β-lactoglobulin.

The conventional way of using free phenolic acids as mediator/bridging agents usually leads to a colored product due to the formation of quinone components in the laccase-catalyzed oxidation reaction. Color formation by laccase-catalyzed oxidation of phenolic compounds has already been utilized to synthesize colorants.[37] However, in cases where phenolic compounds are present to serve the purpose of cross-linking of protein, a colored byproduct might be regarded as an undesirable side effect. So far, most of the relevant studies were based on a phenolic-acid-aided system in order to make laccase work on proteins, and color formation seemed to be inevitable in such a system. The vanillic acid modification of WPI facilitated the laccase-catalyzed cross-linking by directly creating sites for phenoxy radical formation in the protein. The protein-bound radical polymerization resulted in noncolored product. This could broaden the use of laccase in cross-linking applications where color formation is unwanted.

Emulsifying Properties of Van-WPI. The observed improvement of stability of the laccase-treated Van-WPI emulsion would be mainly due to the enhancement of interfacial viscoelastic properties. Cross-linking between the adsorbed protein and free protein molecules in the water phase could increase the length and thickness of the protein layer on the droplet surface and therefore provide stronger steric repulsive force against coalescence in collision. On the other hand, the cross-linking between proteins in the continuous phase may play a role in preventing the emulsion sample from creaming. Previous literature had reported the effect of cross-linking on viscosity of high concentration protein solution.[32] In an emulsion system with high protein content, the extensively cross-linked proteins are expected to form a network that decreases the rate of upward movement of oil droplets and thus slows down the creaming process. In our study where the protein concentration was relatively low, although the cross-linked Van-WPI in the continuous phase could not be enough to form a well-shaped network, a further extended protein structure may also increase of viscosity to some extent and limit the movement of oil droplets better than non-cross-linked proteins.

It is theoretically possible that the cross-links can also be formed between proteins that are adsorbed on adjacent oil droplets, resulting in flocculation. The effect of cross-linking on emulsion stability is largely dependent on the extent of the reaction and the conformation of the cross-linked proteins. Huge extended protein polymers formed by a large extent of cross-linking could impair the stability of emulsions by facilitating droplet aggregation while a proper extent of cross-linking could enhance the emulsion stability. In our study where a complex of oligomerized proteins with different extents of cross-linking was formed (panel c, Figure 4), flocculation or coalescence was not observed in the cross-linked Van-WPI emulsion according to the particle size distribution and confocal microscopy results. Hinz et al. reported that pasteurized cream samples which had high fat content (380 g/L) were treated with transglutaminase and the particle size was stable after the cross-linking reaction despite the condensed system where the distance between oil droplets was closer than in ours.[38] Based on the agreement between these two studies, we assumed that the cross-linking did not occur between droplets but between free proteins in the water phase or free proteins and adsorbed proteins. Actually, Færgemand et al. also speculated that the cross-linking enzyme may work more effectively on nonadsorbed protein substrates.[39] A possible explanation for this could be the electrostatic repulsion between droplets or the adsorption of proteins onto the oil surface hampered their mobility and flexibility for interparticle cross-linking. For future study, it would be worthwhile to investigate the viscosity of the continuous phase and the thickness of the adsorbed protein layer after cross-linking treatment.

Potential Applications of the Vanillic Acid Modification. Laccase has drawn much attention nowadays due to its capability to activate and modify lignin, polymerize sugar beet pectin, or arabinoxylan via their ferulic acid moieties.[40] However, the cross-linking application of laccase is affected by the accessibility of phenolic moieties in these raw materials; for example, in lignin the reactive sites are only partially accessible toward laccase. Especially for protein substrates which do not naturally contain many phenolic groups, the efficiency of laccase-catalyzed cross-linking is very limited. In our study, the cross-linkability of protein substrate was enhanced by the vanillic acid modification which in a sense created a "protein−lignin hybrid". This could expand the utilization of laccase in the field of modification of renewable biopolymers and open ways to create new applications which consist of combinations of different renewable biopolymers. Vanillic acid modified proteins may thus become an interesting starting material for production of complex polymers for various purposes.

ABBREVIATIONS USED

ABTS,2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; DMF, dimethylformamide; EA, emulsifying activity; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; ES, emulsion stability; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MS, mass spectrometry; O/W emulsion, oil in water emulsion; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point; SDS, sodium dodecyl sulfate; sulfo-NHS, *N*-hydroxysulfosuccinimide; TFA, trifluoroacetic acid; WPI , whey protein isolate.

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REFERENCES

(1) Neirynck, N.; Van der Meeren, P.; Lukaszewicz-Lausecker, M.; Cocquyt, J.; Verbeken, D.; Dewettinck, K. Influence of pH and biopolymer ratio on whey protein−pectin interactions in aqueous solutions and in O/W emulsions. *Colloids Surf., A* **2007**, *298*, 99–107.

(2) Schultz, S.; Wagner, G.; Urban, K.; Ulrich, J. High-pressure homogenization as a process for emulsion formation. *Chem. Eng. Technol.* **2004**, *27*, 361–368.

(3) Neirynck, N.; Dewettinck, K.; Van Der Meeren, P. Influence of protein concentration and homogenisation pressure on O/W emulsifying and emulsion-stabilising properties of sodium caseinate and whey protein isolate. *Milchwissenschaft* **2009**, *64*, 36–40.

(4) Khalloufi, S.; Corredig, M.; Goff, H. D.; Alexander, M. Flaxseed gums and their adsorption on whey protein-stabilized oil-in-water emulsions. *Food Hydrocolloids* **2009**, *23*, 611–618.

(5) Lawal, O. S.; Adebowale, K. O. Effect of acetylation and succinylation on solubility profile, water absorption capacity, oil absorption capacity and emulsifying properties of mucuna bean (Mucuna pruriens) protein concentrate. *Nahrung* **2004**, *48*, 129–136.

(6) Van Hekken, D. L.; Strange, E. D.; Lu, D. P. Functional Properties of Chemically Phosphorylated Whole Casein. *J. Dairy Sci.* **1996**, *79*, 1942–1949.

(7) Stevenson, E. M.; Horne, D. S.; Leaver, J. Displacement of native and thiolated β-casein from oil-water interfaces - Effect of heating, ageing and oil phase. *Food Hydrocolloids* **1997**, *11*, 3–6.

(8) Ma, H.; Forssell, P.; Partanen, R.; Seppänen, R.; Buchert, J.; Boer, H. Sodium caseinates with an altered isoelectric point as emulsifiers in oil/water systems. *J. Agric. Food Chem.* **2009**, *57*, 3800–3807.

(9) Hiller, B.; Lorenzen, P. C. Functional properties of milk proteins as affected by Maillard reaction induced oligomerisation. *Food Res. Int.* **2010**,

(10) Caessens, P. W. J. R.; Visser, S.; Gruppen, H.; Voragen, A. G. J. β-Lactoglobulin hydrolysis. 1. Peptide composition and functional properties of hydrolysates obtained by the action of plasmin, trypsin, and Staphylococcus aureus V8 protease. *J. Agric. Food Chem.* **1999**, *47*, 2973–2979.

(11) Agboola, S. O.; Dalgleish, D. G. Enzymatic Hydrolysis of Milk Proteins Used for Emulsion Formation. 2. Effects of Calcium, pH, and Ethanol on the Stability of the Emulsions. *J. Agric. Food Chem.* **1996**, *44*, 3637–3642.

(12) Lakkis, J.; Villota, R. Study on the foaming and emulsifying properties of whey protein hydrolysates. *AIChE Symp. Ser.* **1990**, *86*, 87–101.

(13) Junwen, L.; Tiejing, L.; Xinhuai, Z. Hydrogen peroxide and ferulic acid-mediated oxidative cross-linking of casein catalyzed by horseradish peroxidase and the impacts on emulsifying property and microstructure of acidified gel. *Afr. J. Biotechnol.* **2009**, *8*, 6993–6999.

(14) Onwulata, C. I.; Tomasula, P. M. Gelling Properties of Tyrosinase-Treated Dairy Proteins. *Food Bioprocess Technol.* **2008**, 1–7.

(15) Færgemand, M.; Murray, B. S.; Dickinson, E. Cross-Linking of Milk Proteins with Transglutaminase at the Oil-Water Interface. *J. Agric. Food Chem.* **1997**, *45*, 2514–2519.

(16) Littoz, F.; McClements, D. J. Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase. *Food Hydrocolloids* **2008**, *22*, 1203–1211.

(17) Sharma, R.; Zakora, M.; Qvist, K. B. Characteristics of oil-water emulsions stabilised by an industrial α-lactalbumin concentrate, cross-linked before and after emulsification, by a microbial transglutaminase. *Food Chem.* **2002**, *79*, 493–500.

(18) Liu, M.; Damodaran, S. Effect of transglutaminase-catalyzed polymerization of β-casein on its emulsifying properties. *J. Agric. Food Chem.* **1999**, *47*, 1514–1519.

(19) Lorenzen, P. C. Techno-functional properties of transglutaminase-treated milk proteins. *Milchwissenschaft* **2000**, *55* (12), 667–670.

(20) Flanagan, J.; Gunning, Y.; FitzGerald, R. J. Effect of cross-linking with transglutaminase on the heat stability and some functional characteristics of sodium caseinate. *Food Res. Int.* **2003**, *36*, 267–274.

(21) Hiller, B.; Lorenzen, P. C. Functional properties of milk proteins as affected by enzymatic oligomerisation. *Food Res. Int.* **2009**, *42*, 899–908.

(22) Færgemand, M.; Otte, J.; Qvist, K. B. Emulsifying properties of milk proteins cross-linked with microbial transglutaminase. *Int. Dairy J.* **1998**, *8*, 715–723.

(23) Lantto, R.; Puolanne, E.; Kruus, K.; Buchert, J.; Autio, K. Tyrosinase-aided protein cross-linking: Effects on gel formation of chicken breast myofibrils and texture and water-holding of chicken breast meat homogenate gels. *J. Agric. Food Chem.* **2007**, *55*, 1248–1255.

(24) Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. Multicopper oxidases and oxygenases. *Chem. Rev.* **1996**, *96*, 2563–2605.

(25) Mattinen, M.; Suortti, T.; Gosselink, R.; Argyropoulos, D. S.; Evtuguin, D.; Suurnäkki, A.; De Jong, E.; Tamminen, T. Polymerization of different lignins by laccase. *BioResources* **2008**, *3*, 549–565.

(26) Xu, F. Oxidation of phenols, anilines, and benzenethiols by fungal laccases: Correlation between activity and redox potentials as well as halide inhibition. *Biochemistry* **1996**, *35*, 7608–7614.

(27) Mattinen, M.; Kruus, K.; Buchert, J.; Nielsen, J. H.; Andersen, H. J.; Steffensen, C. L. Laccase-catalyzed polymerization of tyrosine-containing peptides. *FEBS J.* **2005**, *272*, 3640–3650.

(28) Mattinen, M.; Hellman, M.; Permi, P.; Autio, K.; Kalkkinen, N.; Buchert, J. Effect of protein structure on laccase-catalyzed protein oligomerization. *J. Agric. Food Chem.* **2006**, *54*, 8883–8890.

(29) Steffensen, C. L.; Andersen, M. L.; Degn, P. E.; Nielsen, J. H. Cross-linking proteins by laccase-catalyzed oxidation: Importance relative to other modifications. *J. Agric. Food Chem.* **2008**, *56*, 12002–12010.

(30) Bourbonnais, R.; Paice, M. G. Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation. *FEBS Lett.* **1990**, *267*, 99–102.

(31) Selinheimo, E.; Lampila, P.; Mattinen, M.-.; Buchert, J. Formation of protein-oligosaccharide conjugates by laccase and tyrosinase. *J. Agric. Food Chem.* **2008**, *56*, 3118–3128.

(32) Færgemand, M.; Otte, J.; Qvist, K. B. Cross-Linking of Whey Proteins by Enzymatic Oxidation. *J. Agric. Food Chem.* **1998**, *46*, 1326–1333.

(33) Frasconi, M.; Favero, G.; Boer, H.; Koivula, A.; Mazzei, F. Kinetic and biochemical properties of high and low redox potential laccases from fungal and plant origin. *Biochim. Biophys. Acta, Proteins Proteomics* **2010**, *1804*, 899–908.

(34) Labat, E.; Morel, M. H.; Rouau, X. Effects of laccase and ferulic acid on wheat flour doughs. *Cereal Chem.* **2000**, *77*, 823–828.

(35) Kallio, J. P.; Auer, S.; Jänis, J.; Andberg, M.; Kruus, K.; Rouvinen, J.; Koivula, A.; Hakulinen, N. Structure-Function Studies of a Melanocarpus albomyces Laccase Suggest a Pathway for Oxidation of Phenolic Compounds. *J. Mol. Biol.* **2009**, *392*, 895–909.

(36) Lahtinen, M.; Kruus, K.; Boer, H.; Kemell, M.; Andberg, M.; Viikari, L.; Sipilä, J. The effect of lignin model compound structure on the rate of oxidation catalyzed by two different fungal laccases. *J .Mol. Catal. B: Enzym.* **2009**, *57*, 204–210.

(37) Mustafa, R.; Muniglia, L.; Rovel, B.; Girardin, M. Phenolic colorants obtained by enzymatic synthesis using a fungal laccase in a hydro-organic biphasic system. *Food Res. Int.* **2005**, *38*, 995–1000.

(38) Hinz, K.; Huppertz, T.; Kulozik, U.; Kelly, A. L. Influence of enzymatic cross-linking on milk fat globules and emulsifying properties of milk proteins. *Int. Dairy J.* **2007**, *17*, 289–293.

(39) Færgemand, M.; Murray, B. S.; Dickinson, E.; Qvist, K. B. Cross-linking of adsorbed casein films with transglutaminase. *Int. Dairy J.* **1999**, *9*, 343–346.

(40) Riva, S. Laccases: blue enzymes for green chemistry. *Trends Biotechnol.* **2006**, *24*, 219–226.

PUBLICATION IV

Transglutaminase catalyzed cross-linking of sodium caseinate improves oxidative stability of flaxseed oil emulsion

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Transglutaminase Catalyzed Cross-Linking of Sodium Caseinate Improves Oxidative Stability of Flaxseed Oil Emulsion

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ABSTRACT: Sodium caseinate was modified by transglutaminase catalyzed cross-linking reaction prior to the emulsification process in order to study the effect of cross-linking on the oxidative stability of protein stabilized emulsions. The extent of the cross-linking catalyzed by different dosages of transglutaminase was investigated by following the ammonia production during the reaction and using SDS−PAGE gel. O/W emulsions prepared with the cross-linked and non-cross-linked sodium caseinates were stored for 30 days under the same conditions. Peroxide value measurement, oxygen consumption measurement, and headspace gas chromatography analysis were used to study the oxidative stability of the emulsions. The emulsion made of the cross-linked sodium caseinate showed an improved oxidative stability with reduced formation of fatty acid hydroperoxides and volatiles and a longer period of low rate oxygen consumption. The improving effect of transglutaminase catalyzed cross-linking could be most likely attributed to the enhanced physical stability of the interfacial protein layer against competitive adsorption by oil oxidation products.

KEYWORDS: *Sodium caseinate, transglutaminase, cross-linking, emulsion, oxidative stability*

INTRODUCTION

Oxidative stability is an important quality parameter in food emulsions especially in products containing unsaturated fatty acids. Lipid oxidation involves complex radical chain reactions between lipids and oxygen-active species and develops a number of adverse effects on the aroma, flavor, and nutritional value of emulsions.[1] The oxidative stability of an emulsion is dependent on the availability and interaction between reactants: lipids, oxygen, and pro- and antioxidants.[2] Some approaches have been attempted to decrease the oxidative susceptibility and/or to enhance the antioxidant properties of the continuous phase of emulsions, for example, by excluding oxygen from the system, chelating transition metals, or adding antioxidants.[3−5] In the case of a protein stabilized emulsion, the lipid oxidation may be retarded by reinforcing the interfacial protein layer between the oil and the continuous phase against the transfer of oxygen and oxidation products. It has been hypothesized that properties of the interfacial layer such as compactness, thickness, and interfacial rheology are key in influencing the rate of mass transfer to and from the dispersed phase of an emulsion.[6] Emulsion with a thicker interfacial layer of surfactant or protein has been proven to have enhanced oxidative stability.[7,8]

Supply of oxygen may become a limiting factor for the overall oxidation rate. Assuming an efficient transfer of oxygen from the gas to the aqueous phase of the emulsion and a rapid consumption of oxygen in the oil phase in the case of polyunsaturated fatty acids which result in an oxygen gradient across the interface, the diffusion across the layer controls the reaction rate. Real interfacial layers can be mono- or multilayers, but nevertheless, they are very thin. The Fickian diffusion model may not be applicable if the size of the permeant is similar to film thickness.[9] Yet Fickian diffusion has been used to model gas transfer across multilayer interfaces.[10] The system of the present study is further complicated by the viscoelastic nature of the protein layer. In a homogeneous liquid medium, translational diffusion coefficient of solubles is affected by the viscosity of the medium described by the Stokes−Einstein relationship.[11]

The other factors that may affect oxidation are the natural antioxidant activity of the interface forming proteins and electrical charge of the interface.[12,13] Proteins are able to inactivate reactive oxygen species, scavenge free radicals, and chelate transition metals with their histidine, glutamic acid, aspartic acid, and phosphorylated serine and threonine residues.[12] Further on, an electrical charge of the interfacial proteins can influence the affinity of positively charged transition metals to the oil phase. A positively charged interface was found to induce less oxidation because of the electrostatic repulsion between transition metals and the droplet surface.[13]

Transglutaminase introduces inter- or intramolecular cross-links in proteins by catalyzing the acyl transfer reaction between a γ-carboxyamide group of glutamine residues and a ε-amino group of lysine residues.[14] The effect of transglutaminase catalyzed cross-linking on the viscoelastic properties of the interface and on the physical stability of emulsions has been studied earlier,[15−17] but not much information on its effect on oxidative stability is available. Kellerby et al studied the effect of transglutaminase catalyzed cross-linking on lipid oxidation in a sodium caseinate stabilized emulsion.[2] The postemulsification cross-linking was found to take place mainly between the

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adsorbed proteins, resulting in a more cohesive interfacial film as indicated by reduced displacement of the cross-linked interfacial proteins by Tween 20. However, no improvement of oxidative stability was found during the storage for 10 days. For extremely oxygen-sensitive polyunsaturated fatty acids, post-emulsification cross-linking might not be fast enough to retard lipid oxidation, since the oxidation could have deteriorated the oil before any cross-linked interface is formed. Cross-linking before emulsification process could increase both the thickness and the cohesiveness of the interfacial protein layer. Therefore, we assumed that pre-emulsification cross-linking stands a better chance to inhibit lipid oxidation in emulsions.

The aim of this work is to study the possibility of improving the oxidative stability of protein stabilized O/W emulsions by applying a pre-emulsification cross-linking with transglutaminase and whether the possible differences in reaction rates can be linked with oxygen consumption and thus oxygen transfer across the interface. Sodium caseinate, which is known to be reactive toward transglutaminase,[18] was used as the emulsifier in this study.

■ MATERIALS AND METHODS

Materials. Sodium caseinate was obtained from KasLink Foods (Finland) (protein 94%, lactose 0.1%, fat 1%, ash 3.5%), and flaxseed oil was purchased from Elixi Oil Oy (Somero, Finland), where the fatty acid content was the following: 4% 16:0, 3% 18:0, 12% 18:1, 15% 18:2, and 66% 18:3. Microbial transglutaminase Activa MP (TGase) was purchased from Ajinomoto (Japan) and was further purified as described by Lantto et al.[19] The activity of the enzyme was determined by colorimetric hydroxymate method.[20]

Cross-Linking of Sodium Caseinate. The cross-linking of sodium caseinate was performed prior to emulsification. Sodium caseinate was solubilized in boiling Milli-Q water at a protein concentration of 1%, and the temperature of the solution was cooled to room temperature. The pH was adjusted to 7 using 1.0 M NaOH and 1.0 M HCl. Transglutaminase at dosage of 0, 50, 100, 500 nkat/g sodium caseinate was added after the protein was well dissolved. The samples were incubated at room temperature with mild agitation overnight, and then the transglutaminase was deactivated by heating at 90 °C for 10 min. The control sample without transglutaminase was subjected to the same thermal treatment.

Analysis of the Extent of Cross-Linking. Ammonia production was measured using a kit (R-Biopharm AG). The ammonia that was released from the transglutaminase catalyzed reactions reacted with 2-oxoglutarate to form L-glutamate in the presence of glutamate dehydrogenase (GIDH) and reduced nicotinamide dinucleotide (NADH), whereby NADH was oxidized and was determined by its light absorbance at 340 nm after exactly 20 min of reaction. Two replicates were made for each sample. The cross-linking of sodium caseinate with different dosage of transglutaminase was further studied using a 12% SDS gel (BIO-RAD). A mixture with 5 μL of SDS−PAGE loading buffer (Tris-HCl with β-mercaptoethanol and SDS), 5 μL of water, and 5 μL of sample was heated at 98 °C for 10 min and then loaded into each well.

Preparation of O/W Emulsion. The pH of each sample after the enzyme treatment was readjusted to 7 using 1.0 M NaOH and 1.0 M HCl. Then 90% w/w protein solutions were homogenized with 10% w/w flaxseed oil in two steps. A pre-emulsion was prepared using a stirring-type homogenizer (Heidolph Diax 900, Germany) under constant conditions: 2 times, 2 min at 26 000 rpm at room temperature. The main emulsification was performed by loading 110 mL of pre-emulsion into a pressure homogenizer (MicrofluidicsM-110Y, U.S.) at 0 °C and 40 psig (500 bar). The pre-emulsion was circulated in the homogenizer for 10 min, during which it passes through the chamber 30 times. Sodium azide (0.02 wt %) was added to the emulsions to prevent microbial growth. Immediately after the emulsification process, 100 mL of emulsion was transferred into a 1 L glass bottle and stored in the dark at room temperature with stirring at 300 rpm.

Particle Size Determination. Particle size distribution of the emulsions was measured by laser diffraction (Beckman Coulter LS230, CA). After 0, 5, 10, 15, 20, 25, and 30 days of storage, 1 mL of emulsion sample was transferred into an Eppendorf tube and briefly vortexed before each measurement. The measurement was conducted in an optical model with fluid refractive index 1.33 and the sample refractive index 1.46. The pH of Milli-Q water was adjusted to 7 using 0.1 M HCl and 0.1 M NaOH and used as the measuring medium. The volume-surface mean particle diameter (d32) was determined from the particle size distribution of three batches of emulsions. Two measurements of particle size distribution were conducted from each batch.

Peroxide Value Determination. Peroxide value (POV) was determined spectrophotometrically according to IDF standard 74A:1991. Emulsion (0.3 g) was added to 9.6 mL of chloroform/methanol (7:3) mixture. For color formation, 50 μL of both iron(II) chloride and ammonium thiocyanate solutions were added. The sample was briefly vortexed, reacted in the dark for exactly 5 min, and measured at a wavelength of 500 nm. The experiment was repeated three times.

Headspace Analysis. Fresh emulsion samples (2 mL) were transferred into special 6 mL headspace vials and sealed with silicone rubber Teflon caps with a crimper. The samples were stored in the dark at room temperature with stirring at 300 rpm. The products of lipid oxidation in emulsions stored for 0, 6, 12, 18, 24, and 30 days were analyzed using static headspace gas chromatography (Autosystem XL gas chromatograph equipped with an HS40XL headspace sampler, Perkin-Elmer, Shelton, CT; column NB-54, Nordion) according to the method of Frankel et al. with slight modifications.[5] The emulsions were heated at 80 °C for 30 min. The oven temperature was set to 60 °C, and the run time was 10 min. The analysis was carried out based on the results obtained from three replicates of each emulsion sample.

Oxygen Consumption Measurement. Lipid oxidation in emulsions was studied by monitoring the oxygen consumption in a sealed vial with a single-channel oxygen meter (Precision Sensing GmbH, Regensburg, Germany). The vial (1.84 mL) with a small magnet stirrer at the bottom was completely filled with emulsions after 0, 5, 10, 15, 20, 25, and 30 days of storage and capped. The stirring speed during the measurement was 100 rpm. Consumption of the oxygen was monitored at 5 min intervals for 24 h. The consumed oxygen was plotted against time, and the rate of oxygen consumption was calculated as the slope of the linear part of each plot. The unit was presented in millimole of oxygen consumed in 1 L of emulsions per day. The experiment was repeated three times.

■ RESULTS

Cross-Linking of Sodium Caseinate by Transglutaminase. The transglutaminase catalyzed reaction results in formation of ε-(γ-glutamyl)lysine cross-link with concomitant release of ammonia.[14] Sodium caseinate was treated with 0−500 nkat TG/g caseinate, and the amount of the produced ammonia was monitored spectrometrically (Figure 1). The extent of cross-linking reaction, as indicated by the ammonia production, increased with increasing dosage of transglutaminase and was the highest when 500 nkat/g transglutaminase was applied.

The cross-linked proteins were further studied using SDS−PAGE electrophoresis (Figure 2). At a dosage of 50 nkat/g, most of the sodium caseinate stayed as monomers and the intensity of the bands of protein polymers with M_w around 97−206 kDa increased only slightly (lane 3). At a transglutaminase dosage of 500 nkat/g, most of the sodium caseinate was cross-linked into polymers and a band of extensively cross-linked sodium caseinate was observed on the top of the lane (lane 5).

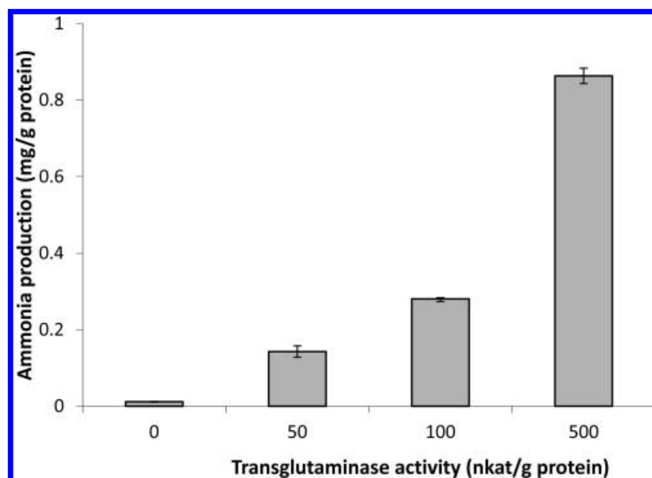

Figure 1. Ammonia produced in the cross-linking reaction between lysyl and glutamyl residues in 1% sodium caseinate solution at different transglutaminase dosages at pH 7 and 20 °C for 20 min. Each point represents the average of two replicates.

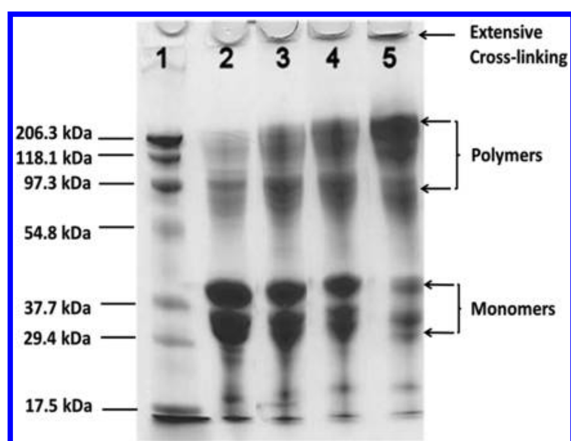

Figure 2. SDS−PAGE of the cross-linked sodium caseiante by transglutaminase. Sodium caseinate was treated with transglutaminase at an enzyme dosage of 50, 100, and 500 nkat/g at room temperature overnight: lane 1, prestained SDS−PAGE standard proteins of 206.3, 118.1, 97.3, 54.8, 37.7, 29.4, and 17.5 kDa migrated from the top to the bottom (BIO-RAD); lane 2, noncross-linked sodium caseinate; lane 3, cross-linked sodium caseinate with 50 nkat/g transglutaminase; lane 4, cross-linked sodium caseinate with 100 nkat/g transglutaminase; lane 5, cross-linked sodium caseinate with 500 nkat/g transglutaminase.

Physical Stability of Emulsions Made of Non-Cross-Linked and Cross-Linked Sodium Caseinate. Emulsions made of the non-cross-linked sodium caseinate (CN) and cross-linked sodium caseinate by 500 nkat/g transglutaminase (TGCN) were prepared under the same homogenization conditions. The lipid oxidation is known to accelerate at the droplet surface, and susbsequently the rate of lipid oxidation increases when more surface area is created with smaller droplet size.[1] Therefore, it is important to investigate the droplet size of CN and TGCN emulsions before discussing the effect of cross-linking. The volume-surface mean particle diameter (d32) of the particles in these two emulsions was followed during the storage time (Figure 3). The pre-emulsification cross-linking did not affect the emulsifying activity of sodium caseinate, since the mean droplet diameter of the fresh TGCN emulsion was the same as that of the CN emulsion (0.16 μm). The physical stability of emulsions was slightly improved by the trans-

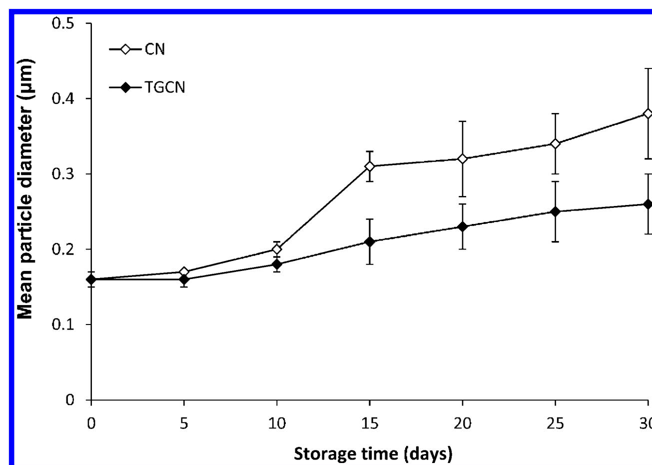

Figure 3. Physical stability of emulsions over 30 days of storage. The emulsions made of noncross-linked and cross-linked sodium caseinate were stored at room temperature in the dark for 30 days. Samples were taken every 5 days, and the volume-surface mean particle diameter (d32) was calculated from volume distribution of three batches of emulsions. Two measurements of particle size distribution were conducted from each batch.

glutaminase treatment. A significant increase of particle size was still observed in both of the emulsions during the storage. After 30 days, the mean droplet diameter increased to 0.26 μm for the TGCN emulsion and 0.38 μm for the CN emulsion because of either droplet flocculation or coalescence.

Effect of Transglutaminase Catalyzed Cross-Linking on the Oxidative Stability of Emulsions. The oxidative stability of the emulsions stabilized by the non-cross-linked sodium caseinate and the cross-linked sodium caseinate was further studied. The emulsions made of sodium caseinate cross-linked by different dosage of transglutaminase exhibited very similar stability against lipid oxidation in the peroxide value measurements (data not shown). Therefore, only the emulsion made of sodium caseinate cross-linked by the highest enzyme dosage (500 nkat/g) was compared with the emulsion with the unmodified protein.

Peroxide value is a measure commonly used to describe the quality of oil, but instead of measuring the rate of hydroperoxide formation, it is measuring the concentration of hydroperoxides in the oil phase, which depends on their decomposition rate in further steps of the reaction. The rate of peroxide decomposition may be of importance in aqueous systems, where it can be catalyzed by the metals present. The peroxide values were measured in order to study the reaction rates (Figure 4). The concentration of fatty acid hydroperoxides stayed at a relatively low level during the first 15 days of storage for both CN emulsion and TGCN emulsion, whereas the formation of peroxides in the CN emulsion accelerated after the induction phase and reached a considerably higher level (386 mequiv/kg oil) after 26 days compared to the TGCN emulsion (50 mequiv/kg oil).

Further, the volatile compounds produced during the storage were analyzed by headspace gas chromatography (Figure 5). Since the flaxseed oil used in this study contains a mixture of different unsaturated fatty acids, there was a complex mixture of secondary oxidation products formed during the lipid oxidation. For this reason, whole chromatograms are shown with two identified peaks for propanal and hexanal, respectively, and other peaks corresponding to the unidentified oxidation

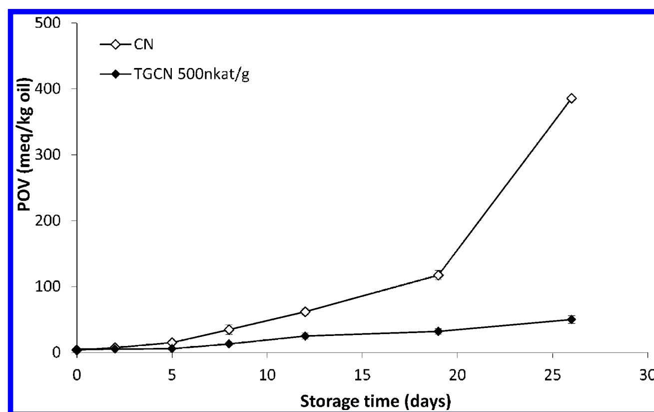

Figure 4. Oxidation measured as peroxide values of flaxseed oil emulsions as stored without light exposure at 20 °C with mixing and large airspace to ensure supply of oxygen to continuous phase.

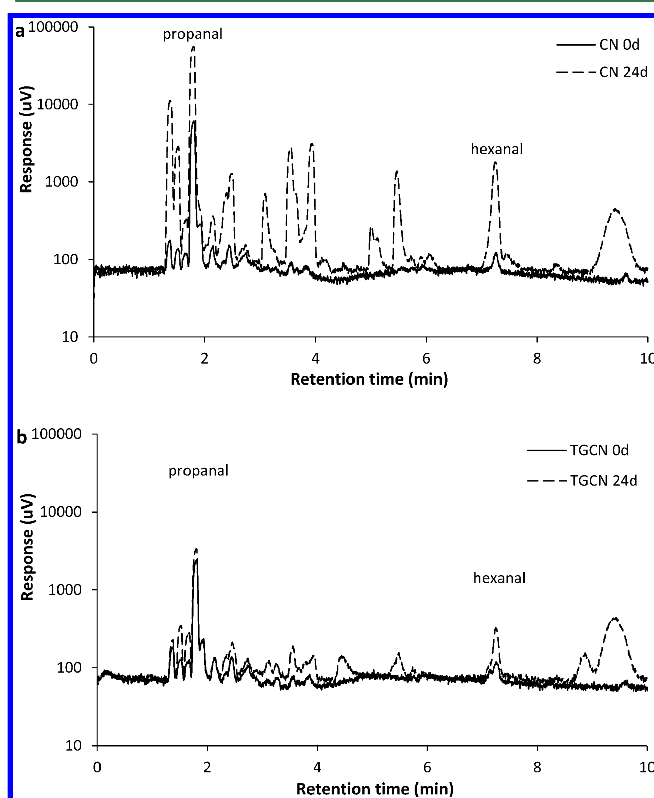

Figure 5. Headspace analysis. The secondary products of lipid oxidation were followed by head space gas chromatography. The whole chromatograms of fresh emulsions prepared with non-cross-linked and cross-linked sodium caseinate and the emulsions after 24 days of storage were presented by plotting the detector response against retention time.

products. The GC analysis was conducted at 0, 6, 12, 18, 24, and 30 days of storage. For fresh CN emulsions, only one sound peak of propanal was detected with peak area 2.8×10^4; the peak area for hexanal was nearly zero. During the following 18 days of storage, a gradual growth was found for the peak area of propanal (1.2×10^5) and hexanal (3900) and no significant change was found for the overall shape of the chromatogram (data not shown). At day 24, the peak area of propanal and hexanal sharply increased to 2.7×10^5 and 1.1×10^4 and a number of other unidentified products were detected (Figure 5a). The production of volatiles in the TGCN emulsion was effectively inhibited. The peak area of propanal and hexanal increased from 1.1×10^4 to 1.6×10^4 and from 0 to 1300, respectively, after 24 days of storage (Figure 5b). The result obtained from the emulsion samples after 30 days of storage showed the same trend: apparent increase for each peak for the CN emulsion and slight increase for the TGCN one (data not shown).

The rate of oxidation was followed by measuring the oxygen consumption in the emulsions with increasing duration of storage (Figure 6). Oxygen consumption is a direct measurement of oxygen transfer from the continuous aqueous phase to the dispersed oil phase and therefore also directly linked with the formation of fatty acid hydroperoxides in the oxygen-consuming step of the lipid oxidation reaction. The emulsions were stored under conditions where large air space and constant stirring allowed quick and sufficient oxygen supply to the aqueous phase so that the oxygen concentration in the emulsion samples for the following oxygen consumption measurement can start from the same saturated level. The oxygen consumption measurement is based on the luminescence quenching by oxygen from the aqueous phase. The measurement is performed by minimizing the air space in the measurement vial. Thus, as oxygen is transferred to the oil phase, its amount in the aqueous phase is simultaneously decreasing, which can be measured as reduced rate of luminescence quenching. The same trend as shown in the peroxide value measurement results was found indicating that the lipid oxidation in the TGCN emulsion was obviously retarded. For the CN emulsion, the lipid oxidation went through an induction phase for the first 10 days of storage. The rate of oxygen consumption increased from 0.34 to 1.01 mmol/(L·day). After this phase, the rate sharply increased to 68.7 mmol/(L·day) for the CN emulsion stored for 30 days, which means the oxygen in the oil phase was rapidly consumed by high concentration of intermediate products of oxidation. As comparison, the oxygen consumption rate in TGCN emulsion was much slower, although a slight increase was observed with time. The rate of oxygen consumption increased from 0.41 to 3.54 mmol/(L·day) after 30 days of storage.

DISCUSSION

The results of oxygen consumption, peroxide value measurement, and headspace analysis showed good correlation with each other. Under the storage conditions in this work, the lipid oxidation was probably in the initiation stage during the first 10 days, as the rate of oxygen consumption in the aqueous phase and the production of peroxides were found to be at a low level (Figures 4and 6). Then the CN emulsion went through the propagation stage of oxidation as seen from the acceleration of peroxide production and a much higher oxygen consumption rate for the next 5−10 days. Similar increase was observed in the oxygen consumption rate of TGCN emulsion after 20 days of storage. Only a small amount of volatiles was produced during this period, since most of the droplets had not reached the termination stage of oxidation yet. Therefore, no significant change in the GC chromatograms was detected in the CN and TGCN emulsions during the first 24 days (Figure 5). Many secondary products of oxidation became detectable by GC only after 24 days of storage. Meanwhile the rate of oxygen consumption and peroxide production were still increasing. This may be explained by the variation of the oxidative stage of oil droplets; some of them may have been oxidized more quickly than the others.

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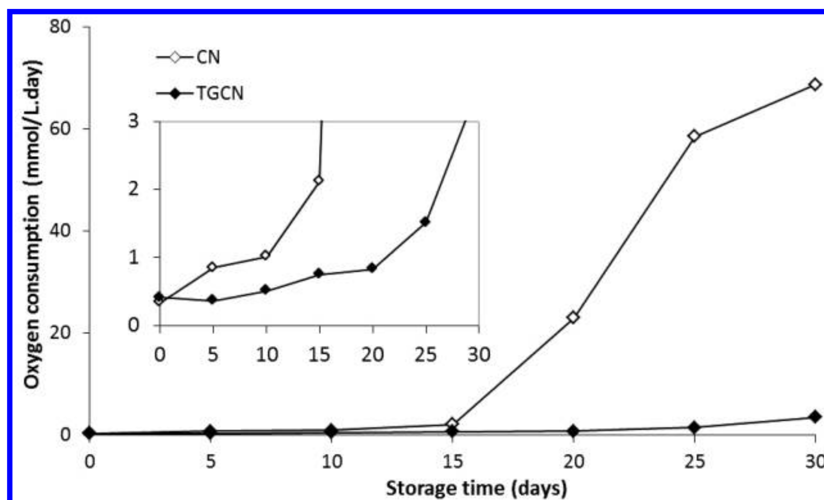

Figure 6. Oxygen consumption measurement. The oxygen consumption in emulsions stored for 0, 5, 10, 15, 20, 25, and 30 days was monitored using a single-channel oxygen meter. The consumed oxygen was plotted against time, and the rate of oxygen consumption was calculated as the slope of the linear part of each plot. The unit is presented in millimole of oxygen consumed in 1 L of emulsions per day.

Besides the above-described properties, particle size was monitored and obtained to be the same for both emulsions during the first 10 days of storage (Figure 3). The particle size increased later to less extent of the TGCN emulsion, indicating that the cross-linking had improved the physical stability of the emulsion. On the other hand smaller particles mean that TGCN emulsion had larger surface area than CN emulsion, which in turn mean larger oxygen flux and higher probability of the oxidation products to interact with the interface.[1]

The primary hypothesis of this work was that by increasing the interactions between the protein molecules that are responsible for forming the interfacial layer, diffusion of oxygen across the layer could be hindered. Overall the initiation stage of oxidation was 10 and 20 days for CN and TGCN emulsions, respectively. Thus, the oil in TGCN emulsion oxidized much more slowly than in CN emulsion. But the rate of oxygen consumption during the initiation period was roughly the same for both emulsions, 0.5−1 mmol/(L·day), considering the small variation in the oxidation rate during the initiation stage. A steady state Fickian diffusion model could be applied for the interface to help the interpretation of the obtained results. The steady state means that the oxygen flux does not depend on time, but it depends on the diffusion coefficient of oxygen within the interface, on the thickness of the layer, and on the concentration gradient across the interface. It is also necessary to assume that oxygen is consumed once it is transported into the oil phase, and thus, the oxygen concentration is zero in the oil phase. This means that oxygen transport across the interface is the rate-controlling step of oxidation.[3,21] Furthermore, as Fickian diffusion is applied, the thickness of the interface has to be considerably larger than the dimensions of the oxygen molecule, which is a reasonable assumption as casein forms multilayers of approximately 10 nm in thickness.[22] Since the particle sizes of the emulsions were almost the same for the first 10 days and later did not much differ from each other, it can be assumed that the effective surface area of the oil droplets was the same during the initiation period of oxidation. Thus, the data can be evaluated based on steady state Fickian parameters. Because the oxygen consumption rates were similar for both emulsions with the same surface area, the retarded oxidation of the oil in TGCN emulsion cannot be explained by the change in diffusion coefficient or increase in the thickness of the interface or decrease of the oxygen gradient across the interface. Apart from oxygen transfer, the cross-linked interfacial layer might inhibit the formation of lipid radicals by limiting the interaction between transition metals in the aqueous phase and the oil phase. Therefore, less hydroperoxides were formed in the TGCN emulsion, supported by the peroxide value measurement (Figure 4), and as a result, the lipid oxidation in the cross-linked emulsion was retarded. The peroxide value measurement is not measuring the rate of hydroperoxide formation but instead the difference between their formation and decomposition rates.[23] Contact with transition metals could also have a role in decomposition of hydroperoxides. This contribution was not supported by the results of the present study, as no accumulation hydroperoxides in the oil phase could be observed for the cross-linked emulsion. A possible explanation for the same oxygen fluxes but delayed oxidation in the case of TGCN emulsion could be due to the different interplay between the reaction products and the interface. The cross-linked sodium caseinate could better protect the interface against adsorption of surface-active compounds produced during the oxidation than CN emulsion and thereby could limit the transfer of these oxidation intermediates into the aqueous phase. Previous study of the displacement of polymerized interfacial protein by surfactant could indirectly provide evidence for the inhibition effect of the cross-linked proteins on the transfer of transition metals and oxidation intermediates across the interface.[24]

A comparable study reported by Kellerby et al. had shown that increasing the cohesiveness of the adsorbed sodium caseinate at the O/W interface did not improve the oxidative stability of emulsions. The transglutaminase cross-linked interfacial sodium caseinate did not influence the diffusion of small pro-oxidant molecules to the core of oil droplets.[2] With the lipids that are both very susceptible to oxidation, the major differences between these two studies were the time point where cross-linking was applied and the storage methods of emulsions. In Kellerby's study, the emulsions were stored at high temperature (55 °C) with limited air space and no agitation. Compared with the present study, where the aqueous phase of the emulsion was kept saturated with oxygen, the conditions were very different and probably responsible for the different results. Also the interfacial protein layers formed may

be structurally different as in the work by Kellerby et al.; interfacial modification was used in comparison to the bulk modification used in the present study. In Kellerby's study, the modification after the formation of emulsions was shown to occur between the adsorbed interfacial proteins rather than between the adsorbed proteins and proteins in the aqueous phase. Therefore, the cross-linking reaction resulted in an increased cohesiveness. In our study, sodium caseinate was cross-linked prior to the emulsification process. For this type of cross-linking, we have previously shown that dilatational elastic modulus of the air−water interface was little affected compared with interfacial modification in the same system.[25] When the protein polymers had adsorbed onto the droplet surface, an interfacial layer with stronger resistance against the penetration of pro-oxidants and against the adsorption of oxidation products compared to that formed with the uncross-linked proteins may form.

This study has shown the potential use of transglutaminase to improve the oxidative stability of protein stabilized emulsions. A remarkable increase in oxidative stability of flaxseed oil emulsions was found when enzymatic cross-linking of emulsifying protein was performed. The increased stability was directly evidenced with delayed propagation stage based on reduced formation of fatty acid hydroperoxides and volatiles and a longer period of low rate oxygen consumption. The exact mechanism of increased stability is yet to be clarified, but at present the most likely explanation is the improved stability of TGCN interface in the presence of oxidized oil. Cross-linking may also have changed the ratio of adsorbed versus nonadsorbed protein, which could increase the thickness of the interfacial protein layer and thus also the amount of antioxidative functional groups of protein localized at the oil−water interface. In future work, it is very interesting and necessary to find out the actual reason for the phenomena reported in this study. It would also be interesting to modify the interfacial proteins in a way that both the electrostatic and steric repulsion against pro-oxidants can be strengthened at the same time. An ideal modified protein could form a thick interfacial membrane with extensive positive charge. Making a protein conjugate with cationic polysaccharide by Maillard reaction could be one possible scenario. Besides, Ma et al. reported modification methods to modify the surface charge of milk protein emulsifiers to positive or negative values at one certain pH. This would provide a good model for studying the effect of interfacial charge on the oxidative stability of emulsions.[26,27]

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Notes

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■ ABBREVIATIONS USED

GC, gas chromatography; GIDH, glutamate dehydrogenase; M_w, molecular weight; NIDH, nicotinamide dinucleotide; O/W, oil in water; PAGE, polyacrylamide gel electrophoresis; pI, iso-electric point; POV, peroxide value; SDS, sodium dodecyl sulfate

■ REFERENCES

(1) McClements, D. J.; Decker, E. A. Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *J. Food Sci.* **2000**, *65*, 1270−1282.

(2) Kellerby, S. S.; Yeun, S. G.; McClements, D. J.; Decker, E. A. Lipid oxidation in a menhaden oil-in-water emulsion stabilized by sodium caseinate cross-linked with transglutaminase. *J. Agric. Food Chem.* **2006**, *54*, 10222−10227.

(3) Marcuse, R.; Fredriksson, P.-O. Fat oxidation at low oxygen pressure. I. Kinetic studies on the rate of fat oxidation in emulsions. *J. Am. Oil Chem. Soc.* **1968**, *45*, 400−407.

(4) Shimada, K.; Muta, H.; Nakamura, Y.; Okada, H.; Matsuo, K.; Yoshioka, S.; Matsudaira, T.; Nakamura, T. Iron-binding property and antioxidative activity of xanthan on the autoxidation of soybean oil in emulsion. *J. Agric. Food Chem.* **1994**, *42*, 1607−1611.

(5) Frankel, E. N.; Huang, S.-W.; Kanner, J.; Bruce German, J. Interfacial phenomena in the evaluation of antioxidants: Bulk oils vs emulsions. *J. Agric. Food Chem.* **1994**, *42*, 1054−1059.

(6) Langevin, D. Influence of interfacial rheology on foam and emulsion properties. *Adv. Colloid Interface Sci.* **2000**, *88*, 209−222.

(7) Silvestre, M. P. C.; Chaiyasit, W.; Brannan, R. G.; McClements, D. J.; Decker, E. A. Ability of surfactant headgroup size to alter lipid and antioxidant oxidation in oil-in-water emulsions. *J. Agric. Food Chem.* **2000**, *48*, 2057−2061.

(8) Hu, M.; McClements, D. J.; Decker, E. A. Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. *J. Agric. Food Chem.* **2003**, *51*, 1696−1700.

(9) Farajzadeh, R.; Krastev, R.; Zitha, P. L. J. Foam film permeability: Theory and experiment. *Adv. Colloid Interface Sci.* **2008**, *137*, 27−44.

(10) Rose, G. D.; Quinn, J. A. Gas transport through supported Langmuir−Blodgett multilayers. *J. Colloid Interface Sci.* **1968**, *27*, 193−207.

(11) Le Meste, M.; Champion, D.; Roudaut, G.; Contreras-Lopez, E.; Blond, G.; Simatos, D. Mobility and Reactivity in Low Moisture and Frozen Foods. In *Water Management in the Design and Distribution of Quality Foods: ISOPOW 7*; Roos, Y. H., Leslie, R. B., Lillford, P. J., Eds.; Technomic Publishing Co, Inc: Lancaster, PA, 1999; pp 267−284.

(12) Elias, R. J.; Kellerby, S. S.; Decker, E. A. Antioxidant activity of proteins and peptides. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 430−441.

(13) Donnelly, J. L.; Decker, E. A.; McClements, D. J. Iron-catalyzed oxidation of Menhaden oil as affected by emulsifiers. *J. Food Sci.* **1998**, *63*, 997−1000.

(14) Folk, J. E.; Finlayson, J. S. The ϵ-(γ-Glutamyl)lysine Crosslink and the Catalytic Role of Transglutaminases. In *Advances in Protein Chemistry*; Anfinsen, C. B., Edsall, J. T., Richards, F. M., Eds.; Academic Press: New York, 1977; Vol. *31* pp 1−33.

(15) Liu, M.; Damodaran, S. Effect of transglutaminase-catalyzed polymerization of β-casein on its emulsifying properties. *J. Agric. Food Chem.* **1999**, *47*, 1514−1519.

(16) Flanagan, J.; Gunning, Y.; FitzGerald, R. J. Effect of cross-linking with transglutaminase on the heat stability and some functional characteristics of sodium caseinate. *Food Res. Int.* **2003**, *36*, 267−274.

(17) Sharma, R.; Zakora, M.; Qvist, K. B. Characteristics of oil-water emulsions stabilised by an industrial α-lactalbumin concentrate, cross-

linked before and after emulsification, by a microbial transglutaminase. *Food Chem.* **2002**, *79*, 493−500.

(18) Han, X.-Q.; Damodaran, S. Thermodynamic compatibility of substrate proteins affects their cross-linking by transglutaminase. *J. Agric. Food Chem.* **1996**, *44*, 1211−1217.

(19) Lantto, R.; Puolanne, E.; Kalkkinen, N.; Buchert, J.; Autio, K. Enzyme-aided modification of chicken-breast myofibril proteins: Effect of laccase and transglutaminase on gelation and thermal stability. *J. Agric. Food Chem.* **2005**, *53*, 9231−9237.

(20) Folk, J. E. Transglutaminase (Guinea Pig Liver). In *Methods in Enzymology*; Herbert Tabor, C. W. T., Ed.; Academic Press: New York, 1970; Vol. *17*, Part 1, pp 889−894.

(21) Marcuse, R.; Fredriksson, P.-O. Fat oxidation at lwo oxygen pressure: II. Kinetic studies on linoleic acid oxidation in emulsions in the presence of antioxidants. *J. Am. Oil Chem. Soc.* **1969**, *46*, 262−268.

(22) Husband, F. A.; Wilde, P. J.; Mackie, A. R.; Garrood, M. J. A comparison of the functional and interfacial properties of β-casein and dephosphorylated β-casein. *J. Colloid Interface Sci.* **1997**, *195*, 77−85.

(23) Osborn, H. T.; Akoh, C. C. Effect of emulsifier type, droplet size, and oil concentration on lipid oxidation in structured lipid-based oil-in-water emulsions. *Food Chem.* **2004**, *84*, 451−456.

(24) Dickinson, E.; Hong, S.-T. Surface coverage of β-lactoglobulin at the oil−water interface: Influence of protein heat treatment and various emulsifiers. *J. Agric. Food Chem.* **1994**, *42*, 1602−1606.

(25) Partanen, R.; Paananen, A.; Forssell, P.; Linder, M. B.; Lille, M.; Buchert, J.; Lantto, R. Effect of transglutaminase-induced cross-linking of sodium caseinate on the properties of equilibrated interfaces and foams. *Colloids Surf., A* **2009**, *344*, 79−85.

(26) Ma, H.; Forssell, P.; Partanen, R.; Seppänen, R.; Buchert, J.; Boer, H. Sodium caseinates with an altered isoelectric point as emulsifiers in oil/water systems. *J. Agric. Food Chem.* **2009**, *57*, 3800−3807.

(27) Ma, H.; Forssell, P.; Partanen, R.; Buchert, J.; Boer, H. Charge modifications to improve the emulsifying properties of whey protein isolate. *J. Agric. Food Chem.* **2011**, *59*, 13246−13253.

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Title	**Role of chemical and enzymatic modifications of milk proteins on emulsion stability/properties** Approaches for more stable protein emulsions
Author(s)	Hairan Ma
Abstract	Milk proteins, sodium caseinate (CN) and whey protein isolate (WPI) are used in food industries as emulsifiers. The stability of an O/W emulsion is dependent on the electrostatic and steric repulsion provided by the interfacial proteins against droplet aggregation or lipid oxidation. Therefore, modifications of the surface charge or the interfacial conformation of protein emulsifiers are expected to enhance their emulsifying properties and emulsion stability. In this present work, sodium caseinate and whey protein isolate were modified by different chemical and enzymatic approaches. The modified proteins were characterized using multiple techniques, and the effect of these modifications on emulsifying properties of proteins and emulsion stability were investigated. Succinylation converts the positively charged amino groups into negatively charged carboxyl groups, lowering the isoelectric point (pI) of protein. The ethylene diamine (EDA) modification worked in the opposite way, leading to an increased pI. The extent of these two modifications was studied using SDS-PAGE and MALDI-TOF mass spectrometry. The pI of succinylated and EDA modified milk proteins was studied using zeta-potential measurement. As a result, the succinylation to full extent altered the pI of CN from 4.2 to 2.7, and the EDA modification shifted the pI of CN and WPI from 4.2 to 9.4 and from 4.9 to 9.5 respectively. The pH stability of emulsion made with the modified milk proteins was monitored by following the increase of particle size during storage. The results suggested that succinylation and EDA modification could enhanced the emulsion stability at pH 4-7 by increasing the electrostatic repulsion between droplets. Regarding the enzymatic modification of milk proteins, the laccase and transglutaminase (Tgase) catalyzed cross-linking were applied on WPI and CN respectively. In order to improve the reactivity of WPI towards the laccase, a vanillic acid modification was carried out to incorporate additional methoxyphenol groups into the protein surface. The cross-linking of vanillic acid modified WPI (Van-WPI) by laccase was studied using SDS-PAGE. The extent of cross-linking of Van-WPI was found to be significantly higher compared to the unmodified WPI and the combination of WPI and free phenolic compound as a mediator. The effect of laccase catalyzed cross-linking on storage stability was investigated by visual observation and confocal microscopy. The post-emulsification cross-linking was proven to enhance the stability of the emulsions prepared with Van-WPI during the storage. The reduced droplet coalescence could be most likely attributed to an extended interfacial protein layer formed via the interaction between the adsorbed proteins and non-adsorbed proteins in the water phase. In contrast with the limited extent of cross-linking of WPI by laccase, CN was extensively cross-linked by Tgase. The physical stability of emulsions was studied by measuring the increase of particle size during storage, and the oxidative stability was evaluated by following the formation of fatty acid hydroperoxides and volatile compounds in different stages of the lipid oxidation. The pre-emulsification cross-linking showed no obvious influence on the physical stability of CN emulsion but significantly improved its stability against lipid oxidation. The improvement of oxidative stability of emulsions could be contributed to a thicker and denser interfacial protein layer and thus increases the amount of anti-oxidative groups located at the interface and provides a stronger barrier against competitive adsorption by oil oxidation products.
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