Food Research International 54 (2013) 1712–1721

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Transglutaminase-induced crosslinking of sodium caseinate stabilized oil droplets in oil-in-water emulsions

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ARTICLE INFO

Article history:
Received 10 June 2013
Accepted 18 September 2013

Keywords:
Transglutaminase
Oil-in-water emulsion
Crosslinking
Critical concentration
Droplet network
Protein

ABSTRACT

The present study established a better understanding of the gelling mechanism of a crosslinking enzyme (microbial transglutaminase) added to sodium caseinate (Na-Cas)-stabilized oil-in-water emulsions varying in droplet concentration and protein content. First, fine dispersed emulsions (5–60% (w/w) Miglyol; 2, 5, and 8% (w/w) aqueous Na-Cas) were prepared using a high pressure homogenizer. Second, microbial transglutaminase was added to Na-Cas-stabilized emulsions (37 °C, 15 h, pH 6.8) to initiate Na-Cas crosslinking. Texture profile analysis and rheological measurements indicated that at low protein concentrations droplet–droplet intercrosslinking occurred above a critical oil volume concentration (c_{oil} > 0.6) yielding particle gels. There, strength of the droplet networks formed depended on oil droplet. At high Na-Cas contents, crosslinking of excess Na-Cas in the aqueous phase occurred and particle-filled continuous gels were formed instead. Theoretical calculations of mean distances between droplets and diffusion coefficients of droplets indicate that a certain probability of contact was required for microbial transglutaminase to be able to crosslink droplets.

1. Introduction

Many foods, pharmaceuticals and personal care products are natural or manufactured emulsion-based materials with complex structures that provide specific functional properties required for their use e.g. rheology, optical appearance, mechanical and chemical stability, or bioactivity (Macierzanka et al., 2011). Emulsions consist of two or more partially or completely immiscible liquids with one liquid being dispersed in the other in the form of small droplets (McClements, 2004a; Walstra, 2003). Droplets in emulsions are typically stabilized by a layer of small molecule surfactants or surface active polymers (e.g. proteins, protein–carbohydrate conjugates, or block copolymers) adsorbed at the oil–water interface (Dickinson, 2012; McClements, 2004a, 2004b). Moreover, droplet characteristics e.g. droplet concentration, droplet size, droplet charge and interfacial composition govern the macroscopic behaviour of emulsions (Dickinson, 2012). At high droplet concentrations, emulsions may attain a viscoelastic behaviour due to the droplets being closely packed. There, droplets are not chemically linked, but are in immediate contact with each other able to transfer stresses across the droplet network. As a consequence, highly concentrated emulsions have an elastic modulus and a yield stress, both properties that are often required to provide texture to foods, adhesion to paints, or efficacy to topical medications. For such networks to form, oil volume fractions must typically exceed 75% and the emulsions are consequently mainly composed of oil (Mezzenga & Ulrich, 2010).

Alternatively, the stabilizing polymers in droplet interfaces may be physically or chemically crosslinked to form a network of droplets. Such systems are referred to as emulsion gels. Of the above mentioned surface-active polymers, proteins are particularly well suited to generate emulsion gels since they possess a wide variety of functional groups that can be covalently crosslinked (Lee, Choi, & Moon, 2006; Tang, Chen, & Foegeding, 2011). In addition to providing the abovementioned functional properties, emulsion gels have shown to be suitable delivery systems for a variety of biologically active compounds such as carotenoids, polyunsaturated fatty acids, phytosterols and vitamins, and have thus of late attracted considerable interest (Chen, Remondetto, & Subirade, 2006; McClements, Decker, Park, & Weiss, 2009; Velikov & Pelan, 2008).

The formation of protein-emulsion droplet networks may be achieved by means of heat treatment, acidification with glucono-δ-lactone (GDL), addition of coagulants such as divalent salts (e.g. $CaCl_2$) or biopolymers (Dickinson, 2012; Tang et al., 2013; Yang, Liu, & Tang, 2013). Moreover, oxidative enzymes including microbial peroxidase, fungal laccase, and bovine plasma monoamine oxidase as well as transglutaminase may be used to chemically crosslink droplets (Dickinson, 1997, 2012; Tang et al., 2011; Yang et al., 2013). Gels formed by non-thermal treatments are referred to as cold set gels and have shown to be particularly well suited to deliver heat-labile bioactives and nutraceuticals (Tang et al., 2011; Yang et al., 2013). Generally, one can distinguish between two structural arrangements in such systems depending on protein and oil droplet concentration: (i) emulsion-filled protein gels in which oil droplets are embedded in a crosslinked protein matrix and (ii) protein-stabilized emulsion droplet gels in which

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http://dx.doi.org/10.1016/j.foodres.2013.09.027

droplets have been crosslinked (Dickinson, 2012). To date, it is not yet completely clear under which conditions one or the other is formed, particularly if a cold setting method such as enzymatic crosslinking is used.

Application of enzymes is considered to be a mild processing technology and can be implemented using conventional processing technologies such as mixing or homogenization (Dickinson, 1997; Minussi, Pastore, & Durán, 2002; Zeeb, Fischer, & Weiss, 2011). Thus, several biomimetic approaches have been carried out to study the ability of enzymes to crosslink biopolymers such as proteins or polysaccharides (Dickinson, 1997; Littoz & McClements, 2008; Zeeb, Gibis, Fischer, & Weiss, 2012a; Zeeb et al., 2011). The most popular enzyme to promote cold-set protein gelation in foods is microbial transglutaminase. This enzyme catalyses the acyl transfer between glutamine and lysine residues in proteins resulting in the formation of both inter- and intramolecular covalent bonds (De Jong & Koppelman, 2002; Motoki & Seguro, 1998). Recently, we conducted a series of studies to crosslink biopolymer layers in multilayered interfaces using enzymes. We demonstrated that application of the oxidoreductase laccase led to formation of crosslinks between beet pectins in a fish gelatin–beet pectin multilayered oil-in-water emulsion, thereby improving its salt, pH, heat, and freeze-thaw stability (Littoz & McClements, 2008; Zeeb et al., 2011, 2012a). Moreover, we showed that a diffusion-driven destabilization of *n*-alkane-in-water emulsions via Ostwald ripening could be retarded by enzymatically crosslinking the interfacial membranes surrounding the oil droplets (Zeeb, Gibis, Fischer, & Weiss, 2012b).

The objective of the present study was to establish a better understanding of the mechanism of action of a crosslinking enzyme added to protein-stabilized oil-in-water emulsions varying in droplet concentration and protein content. We were particularly interested in finding out under which conditions protein–protein crosslinking vs. droplet–droplet crosslinking occurred. To this purpose, a series of oil-in-water emulsions with various oil volume fractions stabilized by sodium caseinate were prepared, and treated with transglutaminase. Sodium caseinate is a mixture of α_{s1}-, α_{s2}-, β-, and κ-casein with approximately 75% of the casein being composed of α_{s1}- and β-casein (Lee et al., 2006; Sanchez & Patino, 2005). Caseinates are known to have a disordered and flexible structure and therefore tend to be more readily crosslinked by transglutaminase than globular and more compact proteins such as e.g. whey proteins (Dickinson & Yamamoto, 1996; Hinz, Huppertz, Kulozik, & Kelly, 2007; Motoki & Seguro, 1998; Sharma, Zakora, & Qvist, 2002a). We hypothesized that a critical oil droplet concentration may be required to promote droplet–droplet crosslinking since the probability of contact governed by the mean surface distance between particles increases as the oil droplet concentration increases. All emulsions were prepared at neutral pH.

2. Materials and methods

2.1. Materials

Sodium caseinate (Na-Cas, #L080512201) was purchased from Rovita GmbH (Engelsberg, Germany). As per manufacturer's specification, caseinate contained ≥88% protein, ≤6% moisture, ≤4.5% ash, ≤1.5% fat, and ≤1% lactose. Sodium caseinate was used without further purification. Miglyol 812N, a medium chain triacylglyceride mixture, was obtained from Sasol Germany GmbH (Brunsbüttel, Germany). It served as a model lipid in the oil-in-water emulsion. Transglutaminase (TGase) was obtained from Ajinomoto Foods Europe SAS (Hamburg, Germany). Z-Gln-Gly (Z-L-Glutaminyl-Glycine, #C6154), iron (III) chloride ($FeCl_3$, #7705-08-0), and trichloroacetic acid (TCA, #76-03-9) were obtained from Sigma-Aldrich Co., (Schnelldorf, Germany). Calcium chloride ($CaCl_2$, #CN93.1, purity ≥ 99.0%) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Double-distilled water was used in the preparation of all samples.

2.2. Transglutaminase activity

Transglutaminase activity was determined according to the method of Folk and Cole (1966) with some modifications using Z-Gln-Gly as a substrate. A mixture of 12 mg/ml Z-Gln-Gly, 100 mM hydroxylamine, 10 mM glutathione (reduced) and 5 mM $CaCl_2$ was prepared in Tris buffer (200 mM, pH 6.0). The reaction cocktail was incubated at 37 °C for 5 min in a thermo mixer. 30 μl enzyme solution (100 mg/ml) was added to initiate the reaction. The reaction was stopped by addition of TCA (12% (w/v), 500 μl) after 10 min. Finally, a $FeCl_3$ solution (5% (w/v), 500 μl) prepared in hydrochloric acid (100 mM) was added to the solution and the absorbance was spectrophotometrically measured at 525 nm (GE, München, Germany). One katal of transglutaminase activity was defined as the amount of enzyme required to form 1 mol γ-monohydroxamate per second at 37 °C and pH 6.0. A transglutaminase activity of 73.1 nkat/ml was measured.

2.3. Solution preparation

An aqueous emulsifier solution was prepared by dispersing 2, 5, and 8% (w/w) sodium caseinate powder in double distilled water containing sodium azide (0.02% (w/w)) as an antimicrobial agent. All solutions were stirred at ambient temperature over night to ensure complete hydration and then adjusted to a pH of 6.8 using 1 M HCl and/or 1 M NaOH.

2.4. Emulsion preparation

Protein concentrations in the continuous phase were kept constant at 2, 5, and 8% (w/w), respectively, whereas the oil droplet concentration was varied between 5 and 60% (w/w). Na-Cas dispersions were mixed with Miglyol using a high shear blender (Standard Unit, IKA Werk GmbH, Germany) for 2 min. The coarse premixes were then passed through a high pressure homogenizer (Avestin, Inc., Ottawa, Ontario, Canada) three times at 1000 bar. Oil-in-water emulsions with higher oil droplet concentrations (50–70% w/w) were prepared by blending oil and emulsifier solutions with a high shear homogenizer (Silent Crusher, Heidolph Instruments GmbH and Co. KG, Schwabach, Germany) for 3 min at 20000 rpm.

2.5. Enzymatic treatment

Caseinate dispersions or emulsions were treated with transglutaminase by mixing them with powdered enzyme (73.1 μkat/l) using a vortexer. A ratio of 1 mg enzyme per 1 g protein solution or emulsion was sufficient to promote crosslinks. Samples were transferred to sealed glass cylinders (height: 100 mm, diameter: 30 mm) and incubated at 37 °C using a temperature controlled cabinet for 15 h and then cooled in a water bath to room temperature. Inversely shown test tubes indicate a transglutaminase-induced sol–gel transition.

2.6. Droplet size distribution

Droplet size distributions were determined by static light scattering (Horiba LA-950, Retsch Technology GmbH, Haan, Germany). Samples were withdrawn and diluted to a droplet concentration of approximately 0.005% (w/w) with water (pH 6.8) to prevent multiple scattering effects. The instrument measures the angular dependence of the intensity of the laser beam scattered by the dilute emulsions and then uses the Mie theory to calculate the droplet size distributions that gave the best fit between theoretical predictions and empirical measurements.