Food Research International 75 (2015) 244–251

Contents lists available at ScienceDirect

Food Research International

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

Cross-linking proteins by laccase: Effects on the droplet size and rheology of emulsions stabilized by sodium caseinate

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ARTICLE INFO

Article history:
Received 28 January 2015
Received in revised form 4 June 2015
Accepted 8 June 2015
Available online 12 June 2015

Keywords:
Emulsion
Laccase
Ferulic acid
Casein

ABSTRACT

The aim of this work was to evaluate the influence of laccase and ferulic acid on the characteristics of oil-in-water emulsions stabilized by sodium caseinate at different pH (3, 5 and 7). Emulsions were prepared by high pressure homogenization of soybean oil with sodium caseinate solution containing varied concentrations of laccase (0, 1 and 5 mg/mL) and ferulic acid (5 and 10 mM). Laccase treatment and pH exerted a strong influence on the properties with a consequent effect on stability, structure and rheology of emulsions stabilized by Na-caseinate. At pH 7, O/W emulsions were kinetically stable due to the negative protein charge which enabled electrostatic repulsion between oil droplets resulting in an emulsion with small droplet size, low viscosity, pseudoplasticity and viscoelastic properties. The laccase treatment led to emulsions showing shear-thinning behavior as a result of a more structured system. O/W emulsions at pH 5 and 3 showed phase separation due to the proximity to protein pI, but the laccase treatment improved their stability of emulsions especially at pH 3. At pH 3, the addition of ferulic acid and laccase produced emulsions with larger droplet size but with narrower droplet size distribution, increased viscosity, pseudoplasticity and viscoelastic properties (gel-like behavior). Comparing laccase treatments, the combined addition of laccase and ferulic acid generally produced emulsions with lower stability (pH 5), larger droplet size (pH 3, 5 and 7) and higher pseudoplasticity (pH 5 and 7) than emulsion with only ferulic acid. The results suggested that the cross-linking of proteins by laccase and ferulic acid improved protein emulsifying properties by changing functional mechanisms of the protein on emulsion structure and rheology, showing that sodium caseinate can be successfully used in acid products when treated with laccase.

1. Introduction

A wide range of food products contains oil-in-water emulsions, e.g., beverages, dressings, soups and sauces. In this case, the small droplets of lipid are dispersed in an aqueous continuous phase. Emulsions are thermodynamically unstable systems, once the droplets tend to break up over time, causing the formation of separated phases (McClements, 2005). In order to improve the kinetic stability of an emulsion, stabilizers and emulsifiers, such as proteins and polysaccharides, can be employed. These biopolymers are widely used as safe additives in the formulation of food emulsions, generally recognized as safe besides having high nutritional value (Chen, Remondetto, & Subirade, 2006).

Proteins form a viscoelastic interfacial layer around the oil droplets, acting as physical barrier against coalescence (Wilde, Mackie, Husband, Gunning, & Morris, 2004). Moreover, the amphiphilic character allows the protein to provide electrostatic and steric repulsive forces against flocculation of the droplets (Dickinson, 2003; Dickinson, 2011).

The milk proteins are mainly separated in two main groups: caseins (~80%) and whey proteins (~20%). Caseins are composed by four main molecular components called α_{s1}-, α_{s2}-, β- and κ-caseins (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999). In the milk, they are organized in sub-micelles, which can be linked together by calcium phosphate, forming the casein micelles that contain κ-casein at the surface, preventing their aggregation by steric and electrostatic repulsion (Walstra, 1999). Sodium caseinate is a salt produced from dissolution of acid-precipitated casein with NaOH (Walstra et al., 1999), resulting in the dissociation of the micellar structure due to the removal of calcium, but maintaining the submicelles (Farrell, Pessen, Brown, & Kumosinski, 1990). This product is widely known by its emulsification and gelation properties (Dickinson, 1999). Caseins are stable to heat, but they aggregate strongly on lower acidic pH, due to the isoelectric point (pI) of their fractions (Parkinson & Dickinson, 2004), which limits their use as emulsifiers.

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http://dx.doi.org/10.1016/j.foodres.2015.06.010
0963-9969/

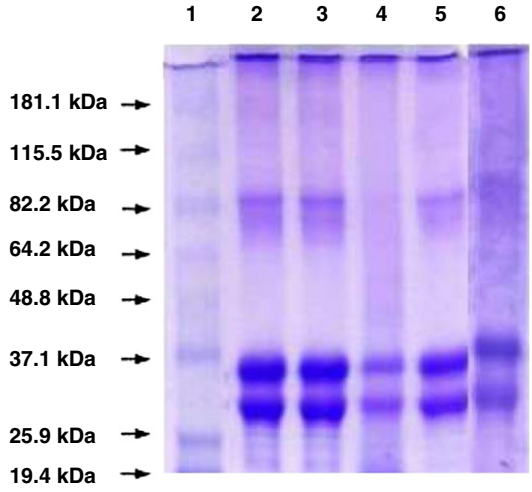

Fig. 1. SDS-PAGE of emulsion composed of sodium caseinate 1% (w/w) at pH 7. (1) Molecular weight marker, (2) control emulsion, (3) emulsion + 10 mM of ferulic acid, (4) emulsion + 10 mM of ferulic acid + 5 mg/mL of laccase, (5) emulsion + 5 mM of ferulic acid and (6) emulsion + 5 mM of ferulic acid + 1 mg/mL of laccase.

The functional properties of proteins for use in food emulsions at varying pH can be improved by crosslinking, which results in increase of stability of emulsions and enhance of resistance to proteolysis (Buchert et al., 2010). This process can be obtained by chemical and/or enzymatic modifications. The advantage of using the enzymatic path instead of chemical modifications is the higher specificity of the enzymatic reactions, minimizing the risks of formation of possible toxic side products (Færgemand, Otte, & Qvist, 1998). Besides of the specificity of enzymatic reactions, the enzymes are considered biodegradable catalyst and require mild reaction conditions (Buchert et al., 2010; Couto & Herrera, 2006), avoiding degradation of labile compounds.

Many enzymes, such as tyrosinase, laccase and transglutaminase, have been used to improve the functional properties of proteins (Cura et al., 2010; Lantto, Puolanne, Kalkkinen, Buchert, & Autio, 2005; Littoz & McClements, 2008; Ma, Forssell, Partanen, Buchert, & Boer, 2011). Laccase is a copper-containing oxidase enzyme that oxidize their substrates by a one-electron removal mechanism leading to the formation of free-radicals. These free-radicals undergo reactions that lead to polymerization. Enzymatic treatment may induce cross-links between proteins both in continuous phase and at interface, protecting oil droplets against coalescence (Paananen, Ercili-Cura, Saloheimo, Lantto, & Linder, 2013). Laccase can act in a considerable range of substrates such as diphenols, polyphenols, different substituted phenols, diamines and aromatic amines (Thurston, 1994). However, proteins such as caseins are reported to be poor substrates for laccase, which may be overcome by the use of substances such as ferulic acid (Paananen et al., 2013). Ferulic acid is a phenolic compound with low toxicity widely used in food and cosmetic industries (Ou & Kwok, 2004). The use of ferulic acid as mediator has been reported to enhance the effects of laccase-catalyzed oxidation of proteins by increasing the accessibility of the reactive amino acids, such as tyrosine and tryptophan, thus improving cross-linking process (Cura et al., 2009; Mattinen et al., 2005; Steffensen, Andersen, Degn, & Nielsen, 2008). Ferulic acid acts as bridging agent in the cross-linking of α-casein, enhancing the laccase-mediated cross-link of the protein (Selinheimo, Lampila, Mattinen, & Buchert, 2008).

Thus, the aim of this work was to evaluate the influence of laccase and ferulic acid on the characteristics of oil-in-water emulsions stabilized by sodium caseinate. The effects of laccase (1 and 5 mg/mL) and ferulic acid (5 and 10 mM) concentrations as well as the emulsion pH (3, 5 and 7) were evaluated.

2. Material and methods

2.1. Material

Laccase enzyme (from *Trametes versicolor*), ferulic acid and sodium azide were obtained from Sigma Chemical Company (St. Louis, USA). Laccase was reported to have 0.51 activity units per mg (AU) of enzyme. Sodium caseinate was prepared from the precipitated casein, obtained from bovine milk (C7078, Sigma Chemical Company, St. Louis, USA). This casein is manufactured by adding acid to milk until its isoelectric point, that is further washed and spray dried (Kinsella & Morr, 1984). The moisture, protein and ash content of casein were 6.51%, 89.64% and 0.84%, respectively. In order to prepare the sodium caseinate, the powder of casein was redissolved in water with the addition of an appropriate amount of sodium hydroxide to restore the neutrality. Soybean oil (Soya, Bunge Alimentos S.A., Brazil) was purchased from a local supermarket and the other chemicals used were of analytical grade (Sigma Chemical Company, St. Louis, USA).

2.2. Solution preparation

Sodium caseinate (Na-CN) stock solution was prepared by dispersing 6% (w/v) casein in deionized water, with gentle magnetic stirring during 3 h at a maximum temperature of 50 °C. The pH of the solution was constantly adjusted to 7 using 10 M NaOH.

Laccase solutions were prepared by dispersing the laccase powder into deionized water, at 1 and 5 mg/mL concentrations. Ferulic acid (AF) solutions were prepared by dispersing of phenolic compound in deionized water, with final concentration of 5 and 10 mM. Both solutions, separately, were gentle magnetically stirred at room temperature to ensure complete dissolution of the materials.

2.3. Laccase treatment and emulsions preparation

Initially, the sodium caseinate (Na-CN) stock solution was diluted to 1% (w/v). Samples with laccase containing (or not) AF were prepared by mixing laccase (1 and 5 mg/mL) and ferulic acid (5 and 10 mM) to the sodium caseinate solutions at room temperature for 2 h (Cura et al., 2009). Emulsions prepared only with sodium caseinate were used as control. All solutions had their pH adjusted with citric acid to obtain solutions with pH 3, 5 or 7, prior the emulsification process.

Oil-in-water (O/W) emulsions were prepared at 25 °C by mixing the soybean oil (30% v/v) with the aqueous phase (70% v/v) containing the sodium caseinate (Na-CN), submitted to the treatment with laccase. To evaluate the effect of the treatments, a control sample was produced only with 1% sodium caseinate. Sodium azide (0.02%, w/v) was added to prevent microbial growth. Firstly, a coarse emulsion was prepared using a rotor–stator device Ultra Turrax model T18 (IKA, Staufen, Germany) (18,000 rpm/4 min). Then fine emulsions were produced by homogenization of coarse emulsions in a two-stage high-pressure valve homogenizer NS1001L2K-PANDA2K (Niro Soavi S.p.A., Parma, Italy). The pressure values in the first and second stages were fixed at 600 bar and 50 bar, respectively (Perrechil & Cunha, 2010). After preparation, no significant variation on pH value was observed. All systems

Table 1
Separation index (% v/v) of emulsions stabilized by Na-CN with ferulic acid (AF) and laccase (LAC).

Systems	pH		
	3	5	7
Control (only Na-CN)	23.2	36.0	–
10 mM AF	4.0	26.4	–
10 mM AF + 5 mg/mL LAC	4.0	48.0	–
5 mM AF	<4.0	25.6	–
5 mM AF + 1 mg/mL LAC	0	52.0	–