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Accessibility of transglutaminase to induce protein crosslinking in gelled food matrices - Influence of network structure

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ABSTRACT

Microbial transglutaminase (mTG) catalyzes the crosslinking of bulk and interfacial proteins, thereby influencing the physical properties of food dispersions. However, little is known about the impact of the spatial distribution of proteins in a gelled matrix on the enzymatic crosslinking. In this study, free and incorporated emulsions were subjected to mTG to induce crosslinking of emulsion interfaces. First, simple caseinate-stabilized emulsions were fabricated by high shear blending (20,000 rpm, 6 min). Second, emulsion samples were embedded into hydrogel beads using an injection technique. Different alginate (0.5–1.5%) and $CaCl_2$ levels (50–500 mM) were used to modulate the hydrogel pore size and number of junction zones. Third, free and incorporated emulsions were mixed with mTG to investigate the diffusion behavior of the enzyme. Results showed that mTG diffused into the beads to an extent of more than 50%. A delay in ammonia release was observed when emulsions were incorporated into hydrogel beads, whereas protein in free emulsions was instantly crosslinked after mTG was added. These results illustrate that the spatial make-up of biopolymers in gelled matrices play a key role on the enzyme accessibility.

1. Introduction

A wide variety of tailor-made encapsulation systems have become essential vehicles to carry and deliver bioactive components in complex food matrices. Typically, suspensions, gels, simple, multiple or multilayered emulsions are used as delivery systems; however, they are known to be thermodynamically unstable. In recent years, enzyme technology was utilized to stabilize and modify physically assembled food structures in order to provide the needed long term stability (Zeeb, Fischer, & Weiss, 2014). As such, this approach is a useful tool to induce covalent bonds in diluted and concentrated emulsions leading to novel properties in terms of stability, digestibility, release kinetics, rheology, and texture. Enzymes such as transglutaminase, laccase, tyrosinase, horseradish peroxidase, and lipoxygenase - just to name a few - have been reported to be excellent texture and structure modifiers (Zeeb et al., 2014).

Microbial transglutaminase is currently one of the most popular enzymes used to modify proteins in terms of solubility, foaming, and emulsification which vastly impacts the functional properties of foods. It catalyzes an acyl transfer between the γ-carboxamide group of glutamine and a primary amine such as the ε-amine group of lysine leading to the formation of isopeptide bonds between proteins. As a product, ammonia is released which could be used as a marker to monitor the enzyme reaction (Kellerby, Gu, McClements, & Decker, 2006). It was demonstrated that transglutaminase promoted the interfacial crosslinking of adsorbed casein, thereby enhancing the physical stability of oil-in-water emulsions (Færgemand, Murray, Dickinson, & Qvist, 1999). Moreover, inter-droplet crosslinking was enzymatically induced to convert liquid emulsions into particulate gels to overcome gravitational separation (Chen, Remondetto, & Subirade, 2006; Dickinson, 1997). Zeeb et al. (2013) showed that droplet-droplet crosslinking occurred above a critical oil volume fraction of 0.6 since mean surface distances between single oil droplets were vastly reduced due to close packing. In addition, the release of bioactive compounds encapsulated into crosslinked emulsions was also modified (Chen et al., 2006).

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However, it was suggested that the accessibility of enzymes to their substrates might play a key role in crosslinking a distinct substrate in a physically assembled structure. As such, it was shown that the rate of laccase-induced oxidation of sugar beet pectin was slower for systems in which emulsions had been homogenized together with pectin, as opposed to when prepared separately. Potentially, the structural make-up of biopolymer substrates at the interface was thought to be a steric hindrance lowering the effectiveness of enzymes for crosslinking (Littoz & McClements, 2008; Zeeb, Fischer, & Weiss, 2011; Zeeb, Gibis, Fischer, & Weiss, 2012). To date, relatively little is known about the effect of the structural assembly of biopolymers that are exposed to an enzymatic crosslinking, particularly in tightly packed systems. For that purpose, emulsion samples were incorporated into hydrogel beads composed of alginate to mimic gelled network structures. Sodium alginate commonly extracted from various brown algae is widely used in food applications due to its thickening and gelling properties. Alginates preferentially interact with multivalent ions such as calcium to form thermo-irreversible gels. In particular, calcium bridges between the carboxyl- and hydroxyl-groups of the guluronic acid backbone are formed, which is usually described as the 'egg-box'-model (Grant, Morris, Rees, Smith, & Thom, 1973). Traditionally, hydrogel formation can be achieved by injecting alginate mixtures dropwise into hardening solutions composed of calcium chloride (Tanaka, Matsumura, & Veliky, 1984).

The objective of the present study was to gain further insights into the crosslinking behavior of enzymes used as structure modifiers in a gelled biopolymer matrix. We hypothesized that the pore size and number of crosslinks in tightly packed systems might impact the diffusion rate of enzymes in such a way that it might be retarded or even completely suppressed. For this purpose, an injection technique was utilized to generate filled hydrogel beads composed of alginate and oil-in-water emulsions stabilized by sodium caseinate as model diffusion system. Filled hydrogel beads were then mixed with transglutaminase under mild stirring to maintain a homogenous suspension and diffusional properties were examined using protein and ammonia measurement techniques. All diffusion experiments were carried out under neutral conditions.

2. Materials and methods

2.1. Materials

Sodium alginate (SA, alginic acid sodium salt #9180.2) was obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Sodium caseinate (SCN, manufacturer's specification: ≥88% protein, ≤6% moisture, ≤4.5% ash, ≤1.5% fat, and ≤1% lactose) was purchased from Rovita GmbH (Engelsberg, Germany). Miglyol 812N, a medium-chain triglyceride oil (MCT), was purchased from Cremer Oleo GmbH & Co. KG (Hamburg, Germany). Microbial transglutaminase (mTG) was obtained from Ajinomoto Foods Europe SAS (Hamburg, Germany). A mTG activity of 73.1 nkat/mL was previously determined (Zeeb et al., 2013). Ammonium chloride (NH_4Cl, ≥99.5%, puriss. p.a., #31107) and sodium azide (NaN_3, ≥99.0%, purum p.a., #71290) were purchased from Sigma-Aldrich (Steinheim, Germany). Bradford reagent Roti®-Quant (#K015.1), calcium chloride ($CaCl_2$, ≥98%, #CN93.2), sodium hydroxide (NaOH, ≥99%, p.a., #6771.5), and hydrochloric acid (HCl, ≥32%, p.a., #P074.4) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Double distilled water was used for the preparation of all samples. All materials were used without further purification.

2.2. Determination of guluronic/mannuronic acid ratio of alginate

For a complete cleavage of the polyuronates, a combination of methanolysis and trifluoroacetic acid (TFA) hydrolysis as described by De-Ruiter, Schols, Voragen, and Rombouts (1992) was used with minor modifications. Briefly, the samples (20 μg) were methanolyzed for 16 h at 80 °C with 500 μL of methanolic HCl. After evaporation, 500 μL of TFA was added and the samples were hydrolyzed for 1 h at 121 °C. After evaporation and coevaporation with ethanol, the samples were redissolved in water and analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using an ICS-5000 System (Thermo Scientific Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA20 column (150 mm × 3 mm i.d., 6.5 μm particle size, Thermo Scientific Dionex). The eluents were 100 mM sodium hydroxide (A) and 100 mM sodium hydroxide + 200 mM sodium acetate (B) and the following gradient was used at 0.4 mL/min and 25 °C. Before every run, the column was rinsed with eluent B and equilibrated with eluent A. 0–30 min, from 100% A to 100% B, 30–42 min, isocratic 100% B. Since mannuronic acid and guluronic acid are not commercially available, they were isolated from a hydrolyzate of the alginate used. In brief, 10 mg of the alginate sample was hydrolyzed with methanolic HCl (2 mL) and TFA (2 mL), evaporated and freeze-dried. The hydrolyzate was fractionated by using an HPLC-ELSD system equipped with a flow splitter and a porous graphitized carbon column (Hypercarb, 100 mm × 4.6 mm i.d., 5 μm particle size, Thermo Fisher Scientific, Waltham, MA, USA). The following gradient composed of double distilled water (A) and double distilled water with 0.1% formic acid (B) was used at 3 mL/min and 70 °C: 0–1 min, isocratic 100% A; 1–8.6 min, linear to 92% A; 8.6–13 min, isocratic 20% A; 13–18 min, isocratic 100% A. Identities and amounts were determined by NMR spectroscopy by using literature data and acetanilide as an internal standard (Heyraud et al., 1996). To determine the guluronic acid/mannuronic acid ratio, monosaccharide concentrations in four methanolyzates were estimated by using an external calibration. A guluronic acid/mannuronic acid ratio of 0.75 (± 0.02) was determined for the alginate sample.

2.3. Solution preparation

Biopolymer stock solutions were prepared by mixing powdered sodium alginate (2.25% w/w) and sodium caseinate (max. 10.7% w/w) with double distilled water. All solutions were stirred over night to ensure complete hydration. Hardening solutions (8, 50, 500 mM $CaCl_2$) were prepared by dissolving $CaCl_2$ in water followed by stirring for at least 30 min. Powdered mTG was dissolved in 8 mM $CaCl_2$ to obtain aqueous enzyme solutions with a concentration of 4 mg g^{-1}. Sodium azide (0.01% w/w) was added to $CaCl_2$ and caseinate solutions to avoid any microbial growth.

2.4. Non-structured aqueous phase: simple oil-in-water emulsions

2.4.1. Emulsion preparation

Simple oil-in-water emulsions (P-O/W) were prepared by blending MCT and caseinate dispersions with a high shear homogenizer (Silent Crusher M with dispersing element 22 G, Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) at 20,000 rpm for 6 min. Different protein-to-oil ratios (1:30; 1:15; 1:8; 1:6; 1:5; 1:4) were tested to reveal emulsions without aggregation or coalescences.

2.4.2. Particle size determination

Particle size measurements were performed using a static light-scattering instrument (Horiba LA-950, Retsch Technology GmbH,