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Chemically modified gliadins as sustained release systems for lysozyme

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ABSTRACT

The aim of this work has been to study the effectiveness of gliadin films cross-linked with cinnamaldehyde as systems for the release of the natural antimicrobial compound lysozyme. Prior to the incorporation of lysozyme, the gliadin film-forming solution was treated with different percentages of cinnamaldehyde as cross-linker, and glycerol was added as plasticizer. The effect of the concentration of the cross-linker on the swelling capacity and kinetics of release of the antimicrobial agent from the protein matrix was evaluated at pH 6.2. The antimicrobial activity of the films was assayed against *Listeria innocua*. The gliadin films cross-linked with cinnamaldehyde incorporating lysozyme preserved their integrity in water. The release rate of the antimicrobial agent was controlled by the reticulation of the protein matrix, thus a greater degree of cross-linking led to slower release of the active agent. Films with a loosely cross-linked structure released a greater amount of lysozyme, exhibiting greater antimicrobial activity.

1. Introduction

Consumers demand for safe, high-quality food products has led to growing interest in the development of active antimicrobial packaging systems. Active packaging can be defined as a type of packaging in which the package, the product, and the environment interact to prolong shelf-life or ensure safety or sensory properties, while maintaining the quality of the product (Suppakul, Miltz, Sonneveld, & Bigger, 2003). The antimicrobial agent can be included in sachets or labels that form part of the packaging system, or they may be included in the package walls, the latter being the more promising development. The amount of active substance released to the packaged product is crucial and must be determined, because high concentrations of a released compound in food could cause sensorial or toxicological problems, whereas low concentrations would not be effective (Conte, Buonocore, Bevilacqua, Sinigaglia, & Del Nobile, 2006). In this connection, a considerable effort is being made to develop sustained active compound release systems for many applications (Buonocore et al., 2004; Chen, Remondetto, & Subirade, 2006; Gemili, Yemenicioglu, & Altinkaya, 2009; Mastromatteo, Conte, & Del Nobile, 2010). Thanks to sustained release systems, it is expected that a minimum inhibitory concentration of the antimicrobial agent will be maintained at the food surface longer than if an equivalent amount of antimicrobial was directly spread or sprayed on the food surface (Min, Rumsey, & Krochta, 2008). Films made from hydrophilic polymers swell in the presence of water which gives rise to the relaxation of polymer matrix favoring the diffusion of the antimicrobial agent to the release medium. A possible way to modulate the release of antimicrobials incorporated into hydrophilic films that has shown good results consists on increasing the cross-linking density of the polymer network and subsequent slow release of the active agent due to a decrease in water swelling (Buonocore et al., 2004).

Gluten is a noteworthy biomaterial for a wide range of technological applications, also as food packaging material (Cornell & Hoveling, 1998). However, gluten is insoluble in water and poorly soluble in ethanol. Gluten proteins are mainly composed of glutenins, a high molecular weight fraction that tend to form large aggregates hindering gluten processability, and gliadins, a family of low molecular weight proteins. Previous studies of gluten proteins have shown that gliadins and glutenins can be exploited separately in order to maximize their range of industrial uses (Hernandez-Munoz, Kanavouras, Ng, & Gavara, 2003). According to this,

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gliadins, which can be extracted with ethanol solutions (60–70%) in which they are highly stable and present excellent film-forming properties (Balaguer, Gomez-Estaca, Gavara, & Hernandez-Munoz, 2011b), represent a more suitable fraction for processing. Gliadin films are very glossy and transparent but have poor mechanical resistance and lose their integrity upon immersion in water (Hernandez-Munoz et al., 2003). Various physical, enzymatic, and chemical treatments can be applied in order to improve their properties owing to the susceptibility to modification of the diverse reactive sites groups present in amino acids (Krochta, 2002). Cinnamaldehyde has recently been shown as a good alternative to other toxic cross-linkers, such as formaldehyde, glutaraldehyde and glyoxal, to improve the physico-chemical performance of gliadin films (Balaguer, M. P., Cerisuelo, J. P., Gavara, R., & Hernandez-Muñoz, P, 2013, Balaguer, M. P., Gomez-Estaca, J., Gavara, R., & Hernandez-Munoz, P., 2011a, 2011b).

Gliadins appear to be highly promising proteins for the development of matrices with a very interesting controlled release potency of diverse molecules (Arangoa, Campanero, Renedo, Ponchel, & Irache, 2001; Balaguer et al., 2014; Duclairoir et al., 1999; Duclairoir, Orecchioni, Depraetere, & Nakache, 2002; Ezpeleta et al., 1996; Gulfam et al., 2012; Kajal & Misra, 2011; Ramteke & Jain, 2008; Stella, Vallee, Albrecht, & Postaire, 1995; Zhou, 2008a; Zhou, 2008b).

Lysozyme (LZ) is a naturally occurring enzyme present in several foods which has specific hydrolytic activity against bacterial cell walls, and it is not toxic to humans (Losso, Nakai, & Charte, 2000). Lysozyme is biocidal against Gram-positive bacteria by hydrolyzing the β-1,4 linkage between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of the cell wall. Consequently, Gram-negative bacteria are not sensitive to lysozyme because their cell walls are poor or lacking in peptidoglycan. Various synthetic or natural polymeric structures have been tested as lysozyme carriers: polyvinyl alcohol beads, nylon pellets and cellulose triacetate films (Appendini, 1997); polyvinyl alcohol films (Conte et al., 2006); plasma-treated polyethylene films (Conte et al., 2008); chitosan (Duan, Park, Daeschel, & Zhao, 2007; Park, Daeschel, & Zhao, 2004); cellulose and polyacrylamide (Datta, Armiger, & Ollis, 1973); whey protein isolate films (Min et al., 2008); alginate and carrageenan (Cha, Choi, Chinnan, & Park, 2002); sodium caseinate (Mendes de Souza, Fernandez, Lopez-Carballo, Gavara, & Hernandez-Munoz, 2010); soy protein (Padgett, Han, & Dawson, 1998); and corn zein (Mecitoglu et al., 2006; Padgett et al., 1998). To the best of our knowledge, there is no previous report on the use of gluten or gliadin films as lysozyme carriers.

The objective of the present work was to characterize the release kinetics of lysozyme from gliadin films cross-linked with cinnamaldehyde as a function of the degree of cross-linking achieved, as well as to evaluate their antimicrobial activity against *Listeria innocua*. The effect of lysozyme incorporation on both the mechanical and barrier properties (water vapor and oxygen) of the films was also evaluated.

2. Materials and methods

2.1. Reagents and bacterial strains

Crude wheat gluten, glycerol, trans-cinnamaldehyde 99%, and lysozyme from chicken egg white were obtained from Sigma (Sigma–Aldrich Química, S.A., Madrid, Spain). Tryptic soy agar (TSA) and Tryptic soy broth (TSB) were purchased from Scharlau (Scharlab S.L., Barcelona, Spain).

L. innocua CECT 910 T was obtained from the Spanish Type Culture Collection (Valencia, Spain) and it was used for testing the antimicrobial activity of lysozyme-gliadin films. Lyophilized *Micrococcus lysodeikticus* ATCC 4698 used for lysozyme release measurement was purchased from Sigma (Sigma–Aldrich Química, S.A., Madrid, Spain).

2.2. Extraction of gliadin-rich fraction

The extraction of the gliadin-rich fraction was carried out according to the method of Hernandez-Munoz et al. (2003). Briefly, 100 g of crude wheat gluten was dispersed in 400 mL of 70% (v/v) ethanol/water, stirred overnight at room temperature, and centrifuged at 5000 rpm for 20 min at 20 °C. The resulting supernatant, containing the gliadin-rich fraction, 15% of protein (w/v), was collected and used as the gliadin film-forming solution.

2.3. Chemical modification of gliadins

In preliminary studies, the use of cinnamaldehyde as a protein cross-linker was found to be strongly dependent on the pH (Balaguer, Borne et al., 2013; Balaguer et al., 2011a, 2011b). Initially, the pH of the gliadin film-forming solution was brought to 2.0 with HCl as the most suitable for polymerization. Then, chemical modification of gliadins was conducted by adding different concentrations of cinnamaldehyde to the film-forming solution, namely 1.5% (G1.5C_pH2), 3% (G3C_pH2), and 5% (G5C_pH2) (g cinnamaldehyde/100 g protein). Glycerol was added as plasticizer at 25% (g glycerol/100 g protein). For films incorporating lysozyme (LZ-GXC_pH2), 10% (g lysozyme/100 g protein) was added. Lysozyme was dissolved into sterile deionized water (15% w/v) using gentle vortexing to obtain a clear colorless solution, and later transferred into the pH-adjusted film-forming solution, maintaining the percentage of ethanol in the film forming solution above 60% to avoid the precipitation of gliadins. The mixture with all the reagents was stirred at room temperature for 30 min.

2.4. Film formation and conditioning

The film-forming solutions were poured onto horizontal flat trays to allow water and ethanol to evaporate. To standardize the concentration of gliadin in the films, a density of 0.01 g of gliadin/cm^2 was used in all cases. The films were dried at 37 ± 2 °C for 24 h. The dried films were peeled off the casting surface and preconditioned in a chamber at 23 ± 2 °C and 53 ± 2% RH for at least 18 h.

Film thickness was measured using a micrometer (Mitutoyo, Kanagawa, Japan) with a sensitivity of ±2 μm. The average film thickness was measured randomly at eight different locations on each film sample.

2.5. Swelling, weight loss, and dimensional stability of antimicrobial gliadin films after immersion in water

Films were cut into circles with a diameter of 2.5 cm ($\emptyset^i$) and conditioned for 10 days in a desiccator containing phosphorus pentoxide (0% RH and 23 °C). Pre-weighed (w^i_{dry}) dry film samples were immersed in beakers containing 10 mL of 0.1 M phosphate buffer at pH 6.2 and 23 °C. At each sampling time films were removed from the buffer, blotted with a Whatman paper and weighed again (w^f_{wet}). The films were placed back into the desiccator until they reached a constant weight, which was used as the final dry weight (w^f_{dry}) of the film. The degree of swelling (S) was evaluated using the following equation:

$$S(\%) = \frac{w^f_{wet} - w^f_{dry}}{w^f_{dry}} \cdot 100 \quad (1)$$