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Mono- and multilayer active films containing lysozyme as antimicrobial agent

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Abstract

Active packaging materials able to release antimicrobial compounds into foodstuff can be used in order to avoid or slow down the bacterial growth during storage. In this work the use of two techniques to control the release of the chosen active compound (lysozyme) from a polymeric material into the foodstuff is proposed: a monolayer cross-linked PVOH film and a multilayer structure made of cross-linked PVOH layers are developed and studied. Lysozyme release tests into water were performed in order to compare the release kinetics from the investigated films. Results suggest that by means of both structures it is possible to control the rate at which lysozyme is released from the PVOH film. The antimicrobial activity of lysozyme released from the investigated films was tested against a suspension of *Micrococcus lysodeikticus*. Results show that the incorporation of lysozyme into PVOH does not lead to a loss of activity of the enzyme.

Keywords: Controlled release; Lysozyme; Mono- and multilayer; Antimicrobial activity

Industrial relevance: The increased use of gently processed foods requires packaging to be an integral part of the preservation concept. Consequently additional antimicrobial activity from the packaging material can aid in shelf life extension.

This paper concentrates on the release rate of lysozyme, a naturally occuring antimicrobial agent (eg. salvia, mothers milk, raw milk), from multilayer films. A comperision of mono- and multilayer films containing lysozyme regarding their effectiveness on *M. lysodeikticus* as target organism was also performed. Both aims were met leading to a controlled release of lysozyme with no loss of activity.

1. Introduction

In recent years controlled release systems became part of a wide category of new food packaging concepts known as “active packaging materials”. Promising active packaging systems are based on the incorporation of antimicrobial substances in food packaging materials in order to control undesirable growth of microorganisms on the surface of food. The antimicrobial compound embedded into the polymer can act with two different kinds of mechanisms. As far as the former is concerned, the preservative is covalently immobilized into the polymer matrix and acts directly from the film when the food is brought in contact with the active material. Regarding the latter, the preservative is embedded into the matrix in the dry state. When the active material is brought in contact with a moist food or a liquid-like food, the preservative is released from the material and acts directly into the food. In both cases the aim of the system is to extend the shelf life of the packaged foodstuff, inhibiting the microbial growth and preserving its sensory properties.

Antimicrobial films intended for food packaging applications have been studied by several authors (Chen, Yeh, & Chiang, 1996; Chung, Chikindas, & Yam, 2001; Chung, Papadakis, & Yam, 2001; Han & Floros, 1998) and the diffusion of antimicrobial compounds from packaging

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materials has been widely reviewed by Han (2000). All these papers deal with the study of active monolayer films. In order to achieve a controlled release of active compound to the food surface, the use of a multilayer film (control layer/active matrix layer/barrier layer) was proposed by Han (1996) and by Floros, Nielsen, and Farkas (2000): the outer layer is a barrier layer which prevents loss of active substances to the environment, the matrix layer contains active substance and has a very fast diffusion, the control layer is the key layer to control the flux of penetration; it has a tailored thickness and diffusivity with respect to the characteristics of microbial spoilage of food product.

On the basis of the previously studied multilayer materials, in this work 2 multilayer films were developed, both composed by two external control layers and by an inner layer containing the chosen active compound, lysozyme. A comparison among the release rate from these multilayer films and from monolayer cross-linked films studied in a previous work by the same authors (Buonocore et al., 2003) was made. The antimicrobial activity of the lysozyme released from the investigated mono- and multilayer films was studied and a comparison among their effectiveness was shown.

2. Materials

The films studied in this work were obtained by cross-linking PVOH (MW = 70,000–100,000, Sigma-Aldrich, Gallarate, Italy). The cross-linking agent was glyoxal (40% aqueous solution, Sigma-Aldrich, Gallarate, Italy) and the catalyser was HCl (37%, Sigma-Aldrich). The active compound was lysozyme from chicken egg white (Sigma-Aldrich, Gallarate, Italy).

3. Methods

3.1. Monolayer film preparation

The active films were obtained by using the following procedure: PVOH (3.25 g) was dissolved into 25 ml of distilled water by keeping the solution into an autoclave at 120 °C for 30 min. The obtained solution was slowly cooled at room temperature. Afterwards lysozyme was added (4 mg/ml), the obtained solution was stirred at ambient temperature until the enzyme was completely dissolved. The obtained solution was cross-linked adding known amounts of glyoxal and 0.2 ml of a 37% aqueous solution of HCl under moderate stirring for 2 h. The obtained solution was cast onto a Plexiglass plate and dried at ambient conditions for 48 h. The obtained films had a length of 16 cm, a width of 12 cm and an average thickness of 120 μm. The thicknesses of the obtained films were measured by means of a Digimatic Micrometer (Mitutoyo, accuracy equal to 0.5 μm). The value of the film thickness was obtained by averaging 100 measurements.

Using this procedure, two monolayer films were obtained, each one having a different cross-linking degree: the first one (which will be referred to as Cross-link A) is obtained adding 0.077% (w/w) of glyoxal; the second one (Cross-link B) is obtained adding 7.7% (w/w) of glyoxal.

3.2. Multilayer film preparation

Multilayer active films were obtained by laying on three different layers: 1) a PVOH film, without active compound, cross-linked with 0.077% (w/w) of glyoxal, 2) a cross-linked/non cross-linked PVOH film containing the active compound (4 mg/ml), 3) a PVOH film, without active compound, cross-linked with 0.077% (w/w) of glyoxal. Each layer was obtained following the procedure described above. Also in this case, the obtained films had a length of 16 cm and a width of 12 cm. For the sake of simplicity, the four investigated films will be named and referred to as reported in Table 1.

3.3. Lysozyme release kinetics

The prepared active films were brought in contact with 250 ml of distilled water at ambient temperature, under moderate stirring. The lysozyme release kinetics were evaluated by monitoring, by means of an HPLC, its concentration in the surrounding solution until an equilibrium value was reached. Each data shown is the average value of three replicates.

3.4. HPLC lysozyme assay

Quantitative determination of lysozyme into the water solution was made by slightly modifying the method proposed by Liao, Brown, and Martin (2001). In particular, lysozyme was determined by means of an HPLC (Agilent Mod. 1100). A C18 Reverse phase column was used (250 × 4 mm, 5 μm) and a gradient elution with water–acetonitrile gradients (1 mL/min) containing 0.1% TFA was used. Typical gradient was 0 to 60% acetonitrile over 20 min, with lysozyme eluting at 10 min. The detection was made at a wavelength of 254 and 225 nm. The calibration curve was constructed for peak area against lysozyme

Table 1
Investigated samples

Sample	Monolayer	Multilayer		
	Containing lysozyme	Layer 1	Layer 2 containing lysozyme	Layer 3
Film A	Cross-link A			
Film B		Cross-link A	No cross-link	Cross-link A
Film C		Cross-link A	Cross-link A	Cross-link A
Film D	Cross-link B			