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Influence of some formulation and process parameters on the stability of lysozyme incorporated in corn flour- or corn starch-based extruded materials prepared by melt blending processing

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ABSTRACT

In order to obtain an antimicrobial biodegradable material, corn flour was extruded with 1% of lysozyme. Since the limited stability of natural preservatives such as lysozyme is a common bottleneck to the elaboration of active biomaterials by melt blending processes, the influence of formulation and of extrusion processing temperature on its residual enzymatic activity was investigated. To assess the contribution of process parameters such as temperature, shear stress and of related formulation parameters such as glycerol and moisture contents, the stability of lysozyme following its extrusion or its thermoforming with plasticized corn starch or thermal treatments in aqueous glycerol solutions was also studied. Increasing glycerol content from 25% to 30% significantly limited inactivation of lysozyme during extrusion, while increasing initial moisture content of the mixture from 14.5% to 28.5% had the opposite effect. These observations open the possibility to prepare active materials retaining more than 60 ± 7% of initial lysozyme activity.

1. Introduction

The interest for biodegradable disposable plastic items has increased over the last decades. This results both from the increase of consumers environmental concern and of crude oil price, since most of plastic items are not biodegradable and based on petrochemicals, respectively. In this context, numerous studies aimed at developing starch- or cellulose-based materials suitable for food packaging. As reviewed by Liu *et al.* [1], starch can be processed into thermoplastic materials in the presence of plasticizers using heat and shear. Most of starch-based materials are made of corn starch. Recently, rigid materials were prepared by extrusion of corn flour in the presence of glycerol and water as plasticizers followed by injection moulding [2,3]. Corn flour contains ∼ 80% starch and 6–8% protein, such as zein, presenting film-forming properties. A clear advantage of using corn flour rather than corn starch is that it is far cheaper since extraction of corn starch generates a high energetic cost. Biodegradable and hygroscopic materials such as corn flour-based materials [2] can be contaminated by food spoilage or food-borne pathogenic microorganisms. In order to prevent this contamination or to obtain an active biodegradable packaging with the aim of extending shelf life of foodstuffs in contact, a possibility is to incorporate food preservatives in these materials. In order not to affect the biodegradability of the corn flour-based materials, natural food preservatives should be preferred. Examination of literature reveals that numerous authors considered the addition of natural antimicrobial molecules in biodegradable materials to obtain biodegradable and antimicrobial materials.

For instance, the addition of antimicrobial plant extracts such as essential oils [4] or of food preservatives such as sorbic acid [5], nisin [6] or lysozyme [7] in biodegradable materials has been proposed. According to Appendini and Hotchkiss [8], traditional ways used for active packaging are: (i) addition of sachets/pads

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containing volatile antimicrobials into packages, (ii) coating or adsorbing antimicrobials onto polymer surfaces (post-thermomechanical processing of polymers operations to obtain antimicrobial packaging materials include for instance enduction [9], soaking [10], spraying [11], or plasma deposition [12] treatments to deposit antimicrobial agents or coatings thereon), (iii) immobilization of antimicrobials to polymers by ionic or covalent linkages, and (iv) incorporation of volatile and non-volatile antimicrobial agents directly into the polymer bulk. For the latter, in most of cases, antimicrobial molecules were directly incorporated in the casting dispersion containing the biopolymer and plasticizers before the drying step necessary to obtain the biomaterials. However, at an industrial scale, most of rigid and flexible food-packaging materials are obtained by a first extrusion (compounding step) followed by either extrusion or injection, respectively. Therefore, as underlined by Del Nobile *et al.* [13], from an industrial point of view, direct incorporation of natural preservatives in the formulation of materials before thermomechanical treatments would generally be preferred. Several authors studied thus the possibility of natural antimicrobials to withstand high temperature and shear stress prevailing during thermomechanical treatments such as extrusion [7,13]. This allowed observing that volatile antimicrobial molecules such as those present in essential oils are generally lost by evaporation during these operations [13]. As foreseen, this confirms that antimicrobial volatile molecules addition to polymeric materials by other ways such as incorporation in a layer of enduction deposited at the surface of materials following their elaboration should be preferred [14]. The possibility to incorporate non-volatile natural antimicrobial molecules such as sorbic acid [15], nisin [16], or lysozyme [7,13,17] has also been studied. Since lysozyme and nisin both have a limited stability above temperatures exceeding ~100 °C, their residual activity was found to namely depend on processing temperature: incorporation into thermoplastics with high melting temperature (>150 °C) resulted in their antimicrobial activity loss [7,13,18]. Denaturation of proteins can also be favoured by a high shear stress during twin-screw extrusion (as reported for lysozyme by Nam *et al.* [7]). Moreover, the thermal stability of an antimicrobial protein such as lysozyme also strongly depends on factors such as pH or water activity [19]: for instance, thermostability of most of proteins is increased in media with a low water content. When preparing thermoplastic corn starch or corn flour, the characteristics of the materials obtained following extrusion both depend on the formulation (namely nature and proportion of plasticizers) and on processing parameters such as shear stress and temperature. In a previous study, Nam *et al.* [7] prepared pea starch extrudates containing 1% (w/w) lysozyme by extrusion in a co-rotating twin-screw extruder with die temperatures varying from 70 to 150 °C and feed moisture contents from 30% to 40% (w/w). Under these conditions, residual activity of lysozyme in pea starch extrudates increased with feed moisture content and decreased with temperature.

In the same way, in this study, 1% (w/w) of lysozyme was incorporated in corn flour or in thermoplastic starch with different percentages of glycerol and extruded or thermoformed at different temperatures. Minimal temperatures of 80 or 90 °C were necessary to get a sufficient destructurization of corn starch and corn flour, respectively, to obtain a homogeneous distribution of lysozyme. In order to avoid total inactivation of lysozyme, less than 120 °C processing temperatures were tested. The initial water content of these blends was tailored by pre-conditioning corn flour or plasticized corn starch in atmospheres with different relative humidities. The assay of the residual activity of lysozyme incorporated in extruded corn flour or thermoformed thermoplastic starch processed at different temperatures in the presence of varying proportions of water and glycerol allowed thus assessing the respective influences of these two plasticizers and of processing temperature on the stability of lysozyme. The sought-after-goal of this study is to get information on the factors allowing to preserve the activity of enzymes, antimicrobial proteins or peptides during thermomechanical treatments increasingly employed to prepare biomaterials. Biomaterials with a sufficient residual antimicrobial activity to be active materials suitable for food preservation could thus be obtained. Moreover, direct addition of other hydrolytic enzymes than lysozyme during extrusion has recently been proposed as an efficient method to hydrolyse biopolymers such as those present in lignocellulosic biomass [20]. In this context, data regarding stability of enzymes during extrusion might also be exploited to develop efficient enzymatic extrusion processes.

Table 1
Composition of the formulations (% w/w) used to prepare corn flour- or corn starch-based extrudates containing lysozyme (corn flour was stored at 23 °C in a 50% RH atmosphere prior to extrusion).

Corn flour or corn starch (%)	Glycerol (%)	Lysozyme (%)
79	20	1
74	25	1
69	30	1

2. Materials and methods

2.1. Preparation of lysozyme-containing corn flour- or corn starch-based materials

Unless stated otherwise, corn flour or thermoplastic corn starch (TPS) were stored at 23 °C in a 50% relative humidity (RH) atmosphere until their moisture content reached equilibrium prior to their use to prepare corn flour- or corn starch-based materials either by extrusion in a three zones single-screw extruder (Scamex, Crosne, France) or by hot press forming with a hydraulic press (Specac, Eurolabo, Paris, France). The screws of Scamex extruder had a length of 220 mm, with a length/diameter ratio of 11.

2.1.1. Preparation of corn flour-based materials

Corn flour, hen egg white lysozyme, and glycerol (99.5% purity) were from Céréгrain S.A. (Bourg en Bresse, France), Sigma-Aldrich (St Louis, MO, USA) and Grosseron S.A. (Saint Herblain, France), respectively. Corn flour-based materials were obtained by extrusion of corn flour, lysozyme, and glycerol (used as a plasticizer) mixtures (Table 1) using Scamex extruder at 90, 100, 110 or 120 °C. The respective resulting temperatures of material were 94, 103, 115 or 124 °C and the residence time in the extruder was about 1–2 min.

2.1.2. Preparation of thermoplastic starch (TPS) and hot-pressed starch films

Native corn starch (Roquette, Lestrem, France) (40% (w/w))–water (60% (w/w)) mixtures were gelatinized by a thermal treatment (60–70 °C) and mixing in a beaker with an Ultraturax disperser-homogenizer (IKA Labotechnik, Staufen, Germany) with a speed ranging between 6000 and 8000 rpm. Then, this starch gel was freeze-dried. Lyophilized starch was mixed with different percentages of glycerol (20, 25, 30% (w/w)) and subsequently stored at 23 °C either in a 50% or 75% RH atmosphere. a_w was measured at 25 °C after equilibration.

The incorporation of lysozyme into 50 mg of starch film was performed by adding 5 μL of a (10%, w/v) lysozyme aqueous solution in order to have a 1% (w/w) lysozyme final content in films. Starch films were prepared by thermoforming the gelatinized starch with a Specac hydraulic press at 100, 110 or 120 °C for 2 min. Films were then immediately cooled at 25–35 °C.

2.1.3. Extrusion of thermoplastic starch-based materials

The incorporation of lysozyme into TPS (containing 30% (w/w) glycerol prepared as previously described) was performed by spraying a 20% (w/w) lysozyme aqueous solution in order to have a final concentration of 1% (w/w) lysozyme in TPS. TPS and lysozyme were mixed and either stored at 23 °C in a 50% or 75% relative humidity (RH) atmosphere or directly extruded: TPS and lysozyme were extruded at 80, 90 or 100 °C. The resulting temperatures of material were 83, 93 and 103 °C, respectively. The a_w value at 25 °C of each mixture was measured before extrusion with an a_w-meter instrument (Fast-1, GBX, Bourg de Péage, France). The rotation speed of the extruder screw was set at 50 rpm which resulted in an approximate residence time of ~55–60 s.

2.2. Extraction of lysozyme from materials by enzymatic hydrolysis

Lysozyme was extracted from corn flour or TPS-based materials and from hot-pressed gelatinized starch films by enzymatic hydrolysis according to the following protocol: corn flour- and starch-based materials were crushed with a Polymixis PX-MFC 90D apparatus (Kinematica AG, Lucerne, Switzerland) set at a speed of