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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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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  $60 \pm 7\%$  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  $\sim 100^\circ\text{C}$ , their residual activity was found to namely depend on processing temperature: incorporation into thermoplastics with high melting temperature ( $>150^\circ\text{C}$ ) 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  $150^\circ\text{C}$  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  $90^\circ\text{C}$  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  $120^\circ\text{C}$  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

**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  $23^\circ\text{C}$  in a 50% RH atmosphere prior to extrusion).

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

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.

## 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  $23^\circ\text{C}$  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 Cérégrain 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  $120^\circ\text{C}$ . The respective resulting temperatures of material were  $94$ ,  $103$ ,  $115$  or  $124^\circ\text{C}$  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 ( $60$ – $70^\circ\text{C}$ ) and mixing in a beaker with an Ultraturax disperser-homogenizer (IKA Labortechnik, 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  $23^\circ\text{C}$  either in a 50% or 75% RH atmosphere.  $a_w$  was measured at  $25^\circ\text{C}$  after equilibration.

The incorporation of lysozyme into 50 mg of starch film was performed by adding  $5\ \mu\text{L}$  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  $120^\circ\text{C}$  for 2 min. Films were then immediately cooled at  $25$ – $35^\circ\text{C}$ .

#### 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  $23^\circ\text{C}$  in a 50% or 75% relative humidity (RH) atmosphere or directly extruded: TPS and lysozyme were extruded at  $80$ ,  $90$  or  $100^\circ\text{C}$ . The resulting temperatures of material were  $83$ ,  $93$  and  $103^\circ\text{C}$ , respectively. The  $a_w$  value at  $25^\circ\text{C}$  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  $\sim 55$ – $60$  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

5000 rpm for 30 s. 100 mg mL<sup>-1</sup> of crushed material suspensions were prepared in sodium phosphate buffer (0.1 mol L<sup>-1</sup>, pH 7). An enzymatic preparation, containing  $\alpha$ -amylase and amyloglucosidase from *Aspergillus niger* (Hazyme DCL, DSM Food Specialties, Seclin, France) was then added to the desired final concentration (50 U mL<sup>-1</sup>) in order to liberate the lysozyme molecules embedded in corn flour- or starch-based materials. Units of  $\alpha$ -amylase or amyloglucosidase (U) are defined as the amounts of enzyme that liberate from starch in 3 min 1 mg of glucose or 1 mg of maltose, respectively. The tubes containing the reaction mixtures were incubated at 50 °C in a controlled temperature incubator (optimal temperature for enzymes activity) under rotary shaking (6 rpm) for 4 h. The reaction mixtures were then immediately centrifuged at 4000  $\times$  g for 10 min at 4 °C to remove insoluble particles [2]. The lysozyme enzymatic activity of the supernatant was assayed as follows.

### 2.3. Assay of lysozyme enzymatic activity

The enzymatic activity of lysozyme was assayed at 25 °C with a turbidimetric assay. Lysozyme substrate was a *Micrococcus lysodeikticus* cell suspension (0.26 mg mL<sup>-1</sup>) (M3770, Sigma Aldrich) in 0.1 mol L<sup>-1</sup> sodium phosphate buffer (pH 7.0). 100  $\mu$ L of the lysozyme solution was added to 2.9 mL of *M. lysodeikticus* cell suspension. Turbidity was measured for 2 min at 450 nm using a spectrophotometer (Jenway 6300, Essex, United Kingdom). The initial slope of the 450 nm absorbance decrease as a function of time was used to estimate the concentration of active enzyme. One unit (U) of lysozyme activity was defined as 0.001 decrease in 450 nm absorbance in 1 min at 25 °C [21]. Lysozyme activity assays were performed 4 times for each sample. The residual activity of lysozyme in each hydrolysed granule or material was calculated as the ratio between lysozyme activity assayed and the corresponding activity of the quantity of lysozyme initially added.

### 2.4. Thermal stability of 1% (w/v) lysozyme solutions

The enzyme solution was prepared by dissolving 1% (w/v) lyophilized lysozyme powder in distilled water. The pH of solution was adjusted to 7 by addition of 1 mol L<sup>-1</sup> NaOH solution. The thermal stability of lysozyme was studied in solutions adjusted to 7 since the pH of corn flour- or corn starch-based materials incorporating lysozyme was close to 7. The  $a_w$  values at 25 °C of solutions containing glycerol were measured with a FA-ST/1  $a_w$ -meter (GBX, Bourg de Péage, France). 2 mL tubes filled with 1% (w/v) lysozyme solutions with or without glycerol were then heated for 3 min at 60, 90, 100, 110, or 120 °C either in a water or oil bath. The tubes were then immediately cooled in an ice-water bath and lysozyme residual activity subsequently assayed.

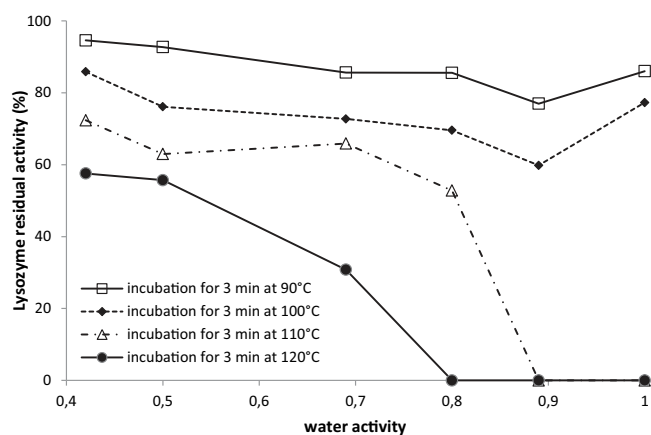
### 2.5. Statistical analysis

All experiments were performed using at least three samples (triplicates). The results presented are the averages and standard deviations that were calculated from these triplicates. Statistical differences between samples were calculated using Fisher's tests with a significance level of  $P > 0.05$  (Statgraphics centurion XV software, 16.1.18 version, Sigmaplus, Levallois-Perret, France).

## 3. Results and discussion

### 3.1. Effect of glycerol on the thermal inactivation of lysozyme

A stabilizing effect of polyols like glycerol on thermal inactivation of lysozyme has been reported by numerous authors [22–24]. It has been proposed to result mainly from its depressing effect on water activity ( $a_w$ ) [25]. Indeed, water activity measures the energetic availability of water and its mobility and its penetration in globular proteins is one of the factors favoring their denaturation. Therefore, lysozyme was solubilized in aqueous solutions containing from 0% to 82% (w/w) glycerol (from which pH was adjusted to 7.0 to be close to the pH of corn flour- and TPS-based materials) to get lysozyme solutions with  $a_w$  values ranging from 1.0 to 0.42 at 25 °C. The corresponding RH at equilibrium values were thus in the same range than in the atmospheres for storage of corn flour before extrusion at temperatures ranging from 90 to 120 °C for about 2 min. Therefore, all these lysozyme solutions with varying glycerol contents were incubated for 3 min either at 90, 100, 110 or 120 °C and their residual enzymatic activity was assayed (Fig. 1). Interestingly, it was observed that a glycerol concentration corresponding to an  $a_w$  value of 0.5 better protected lysozyme from thermal inactivation at temperatures comprised between 90 and 120 °C than lower glycerol concentrations corresponding to  $a_w$  values exceeding 0.5. The higher the temperature, the higher

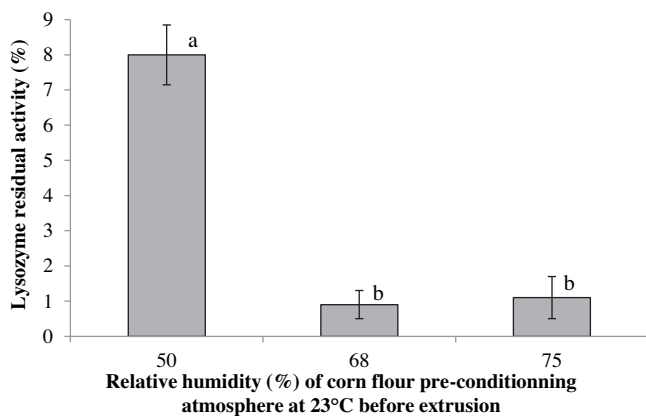


**Fig. 1.** Effect of temperature of heat treatment and of water activity ( $a_w$ )<sup>\*</sup> on the activity of diluted lysozyme solution (1% (m/v)) at pH 7. <sup>\*</sup> $a_w$  at 25 °C values of lysozyme aqueous solutions were fixed by addition of glycerol at different concentrations.

this stabilizing effect: for instance, following a thermal treatment for 3 min at 120 °C in a solution with a higher than 0.8  $a_w$  (i. e. containing less than 50% (w/w) glycerol), lysozyme was totally inactivated while when this solution had an  $a_w$  of 0.42 (i.e. containing 82% (w/w) glycerol), it retained 60% residual activity. This is consistent with Wimmer *et al.* [26] observation that increasing sorbitol concentration in aqueous solution from 0 to 1 mol L<sup>-1</sup> increased denaturation temperature of lysozyme from 77 to 81 °C. These results were explained by considering that polyols addition to aqueous solutions of proteins results in the formation of a protein–water–polyol system instead of a protein–water system. The interactions between proteins and water can thus be affected in different ways by the presence of polyols: (i) on one hand, polyols can be excluded from certain non-polar sites of the surface of proteins and contact of these sites with water would thus be favoured (“solvophobic” effect), (ii) on the other hand, polyols could be attracted by certain polar sites present on the surface of proteins.

### 3.2. Influence of initial moisture content in the formulation of corn flour-based granules incorporating lysozyme on its stability to extrusion

To investigate the effect of water content in “corn flour (74% w/w)–glycerol (25% w/w)–lysozyme 1% (w/w)” mixtures on the stability of lysozyme to thermomechanical processing, corn flour–glycerol mixtures were stored in atmospheres at different relative humidities (RH) before extrusion (Fig. 2). After extrusion at 90 °C, the lower the initial water content (i.e. 14.5%), the higher the lysozyme residual activity (i.e. 8 ± 1%). In the range of moisture contents tested, water promoted the inactivation of lysozyme. This observation may seem contradictory with Nam *et al.* [7] who reported that the extrusion at 70 °C of pea starch containing lysozyme caused inactivation of this enzyme when the water content was low. However, it should be noted that (i) the conditions of temperature and shear stress during extrusion were different, (ii) the water contents also differed (from 30% to 40% and from 14.5% to 28.5% before extrusion in their study and in the present study, respectively) and principally (iii) these authors did not use glycerol in addition to water as a plasticizer. Water and glycerol properties such as absorption of heat, transition to the vapor state, interactions with corn flour and effect on the viscosity of the corn flour–plasticizers mixture during extrusion strongly differ. Moreover, water and glycerol cannot be considered independently for instance because glycerol lowers the energetic availability of water.



**Fig. 2.** Residual activity of lysozyme\* in corn flour-based materials prepared by extrusion at 90 °C of corn flour–glycerol–lysozyme mixtures. The initial water content of these mixtures was 14.5%, 21.8% or 28.5% depending on the relative humidity of their storage atmosphere at 23 °C: 50%, 68% or 75%, respectively. Corn flour, glycerol, and lysozyme contents in mixtures were 74% (w/w), 25% (w/w) and 1% (w/w), respectively [Data with different letters are significantly different (Fisher's test,  $P < 0.05$ )]. \*To allow the assay of its residual enzymatic activity, lysozyme was extracted from corn flour-based materials following hydrolysis of corn starch (the main corn flour constituent) by an amyolytic enzymes preparation as described in Section 2.

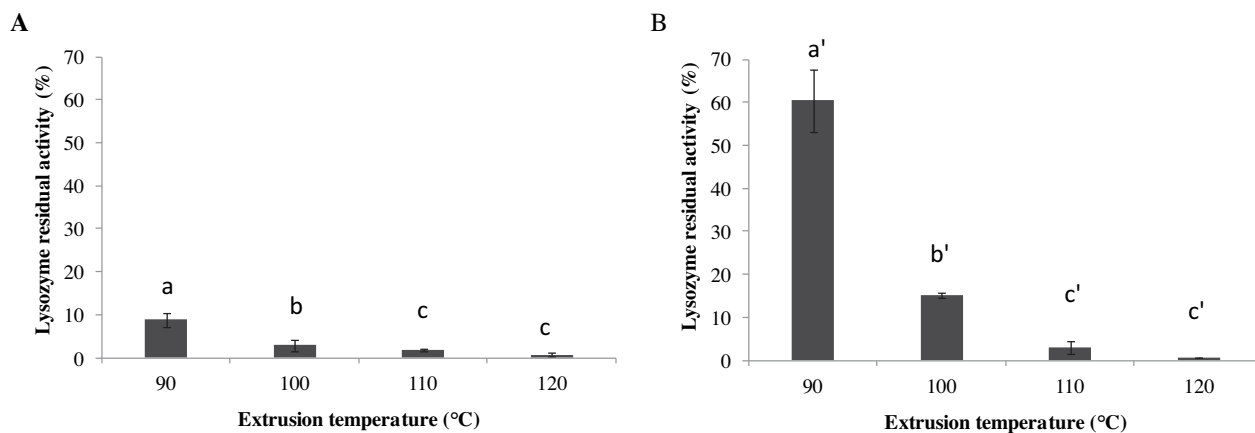
All this probably explains the observed differences regarding the effect of water on the inactivation of lysozyme during extrusion.

### 3.3. Influences of extrusion temperature and glycerol content in the formulation of corn flour-based granules incorporating lysozyme on its stability

The effect of extrusion temperature on residual activity of lysozyme was studied with corn flour–glycerol mixtures stored at 50% RH prior to extrusion. Moreover, since glycerol, besides its plasticizer role, also likely affects lysozyme stability during extrusion, the effect of extrusion temperature on its stability was investigated in materials made of corn flour–glycerol mixtures containing either 25% (w/w) (Fig. 3A) or 30% (w/w) (Fig. 3B) glycerol. As expected, increasing the extrusion temperature from 90 to 120 °C resulted in an increased inactivation of lysozyme: following extrusion at 120 °C, lysozyme was almost completely inactivated (less than 1.5% residual activity). Interestingly, the increase of glycerol content from 25% to 30% (w/w) in corn flour–glycerol mixtures significantly

increased the residual activity of lysozyme following extrusion at 90 and 100 °C: the residual activity of lysozyme following extrusion at 90 °C in materials made from formulations containing 30% (w/w) glycerol was  $60 \pm 7\%$  (against only  $8 \pm 2\%$  in those made of 25% (w/w) glycerol formulations, as previously stated). This increased stability of lysozyme in the presence of 30% (w/w) glycerol can also be illustrated by observing that the residual activity of lysozyme after extrusion at 100 °C with 30% (w/w) glycerol ( $16 \pm 1\%$ ) was higher than following extrusion at 90 °C with 25% (w/w) glycerol ( $8 \pm 2\%$ ). This clearly shows that the stability of lysozyme during extrusion highly depends on corn flour–glycerol–water proportions in the formulation. Relevant proportions might open the possibility to integrate this antimicrobial protein in corn flour-based materials to obtain antimicrobial materials prepared by thermomechanical processes commonly employed in food packaging industry, such as extrusion.

While the increased thermal stability of lysozyme in aqueous solutions of polyols is well documented, studies dealing with its thermal stability in the presence of starch (i.e. the main component of corn flour) or during extrusion are scarce. Denaturation and thus inactivation of lysozyme during extrusion is likely also due to other factors than thermal denaturation enhanced by water: the contribution of surface denaturation due to shear stress can for instance not be excluded. Moreover, glycerol and water, besides modulating the stability of lysozyme, are plasticizers which affect differently the viscosity of the corn flour–water–glycerol–lysozyme mixtures during extrusion. Another important difference between glycerol and water concerns their heat absorption and transition to vapor state properties. Absorption of heat by water during extrusion leads to a significant water evaporation following extrusion. This is illustrated by the simultaneous decrease of moisture content and water activity measured following extrusion (Table 2). Even after extrusion at the lowest set temperature employed within this study (i.e. 90 °C), moisture content of a 70% (w/w) corn flour–30% (w/w) glycerol mixture stored for 72 h at 23 °C in a 50% RH atmosphere decreased from  $18.3 \pm 0.1\%$  before extrusion to  $7.9 \pm 0.1\%$  following extrusion. This important water evaporation induced a decrease of  $a_w$  measured at 25 °C from  $0.46 \pm 0.01$  (a value close to 0.5, i.e. the expected  $a_w$  value at equilibrium in a 50% RH atmosphere) to  $0.32 \pm 0.01$ . In Table 2,  $a_w$  and moisture contents before and after extrusion at 90 °C of 70% (w/w) corn flour–30% (w/w) glycerol mixtures stored for 72 h at 23 °C in 44%, 50% or 68% RH atmospheres are reported. It is clear that the most important moisture content and  $a_w$  reductions were observed following extrusion of mixtures also having the highest initial moisture contents and  $a_w$



**Fig. 3.** Lysozyme residual activity (as a function of extrusion temperature (90, 100, 110 or 120 °C) and glycerol content ((25% (A) or 30% (B) glycerol). The corn flour–glycerol mixtures were stored at 23 °C in a 50% RH atmosphere before extrusion [Data with different letters are significantly different (Fisher's test,  $P < 0.05$ )]. \*To allow the assay of its residual enzymatic activity, lysozyme was extracted from corn starch-based materials following hydrolysis of corn starch by an amyolytic enzymes preparation as described in Section 2.

**Table 2**  
Moisture content and water activity ( $a_w$ ) at 25 °C of corn flour plasticized with 30% (w/w) glycerol after 72 h of storage at 23 °C in an atmosphere at 44%, 50% or 68% RH (i.e. initial moisture content and  $a_w$ ) and of the corresponding materials after extrusion at 90 °C (i.e. final moisture content and  $a_w$ ).

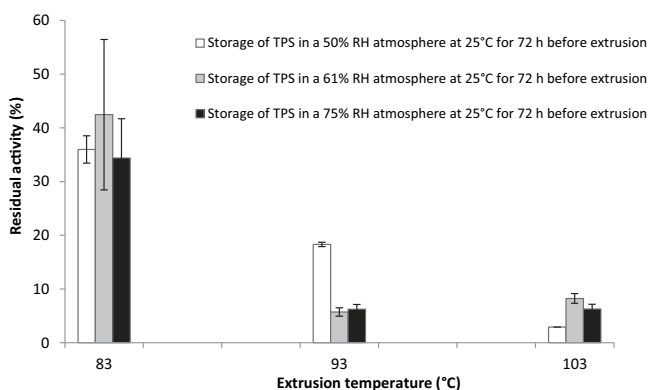
RH of atmosphere of storage at 23 °C (%)	Initial $a_w$ (25 °C)	Initial moisture content (%)	Final $a_w$ (25 °C)	Final moisture content (%)
44	0.46 ± 0.01	14.7 ± 0.1	0.34 ± 0.01	8.9 ± 0.1
50	0.45 ± 0.01	18.3 ± 0.1	0.32 ± 0.01	7.9 ± 0.1
68	0.63 ± 0.01	26.0 ± 0.1	0.53 ± 0.01	13.4 ± 0.1

**Table 3**  
Residual activity of 1% (w/w) lysozyme after extrusion in a corn flour or corn starch formulation, thermoforming in corn starch or storage in water at set temperatures ranging from 80 to 120 °C.

Set temperature <sup>a</sup>	Residual activity of lysozyme (%)			
	Aqueous solution adjusted at pH 7	Formulations with 30% glycerol		
		pre-equilibrated at 23 °C in a 50% RH atmosphere		
	Water Duration = 3 min	Extruded corn flour Duration = 1–2 min	Extruded corn starch Duration = 55–60 s	Thermoformed corn starch Duration = 2 min
80 °C	n.d.	n.d.	39.3	n.d.
90 °C	86.0	60	18.3	n. d.
100 °C	77.3	15	2.9	47.1
110 °C	<0.1	2.6	n.d.	18.3
120 °C	<0.1	0.6	n.d.	7.2

n.d.: Not determined.

<sup>a</sup> Set temperature: During corn flour formulations extrusion, the resulting temperatures corresponding to set temperatures of 90, 100, 110 or 120 °C were 94, 103, 115 or 124 °C, respectively. During corn starch formulations extrusion, the resulting temperatures corresponding to set temperatures of 80, 90 or 100 °C were 83, 93 or 103 °C, respectively.



**Fig. 4.** Effect of extrusion temperature and relative humidity at 23 °C on the residual activity of lysozyme incorporated in extruded TPS formulated with 30% (w/w) glycerol. <sup>a</sup>To allow the assay to its residual enzymatic activity, lysozyme was extracted from corn starch-based materials following hydrolysis of corn starch by an amylolytic enzymes preparation as described in Section 2.

values: that is the highest free water contents (free water in food and biological systems requires a lower energy for its evaporation).

#### 3.4. Influence of extrusion temperature and initial moisture content in the formulation of thermoplastic corn starch (TPS)-based blends incorporating lysozyme on its stability

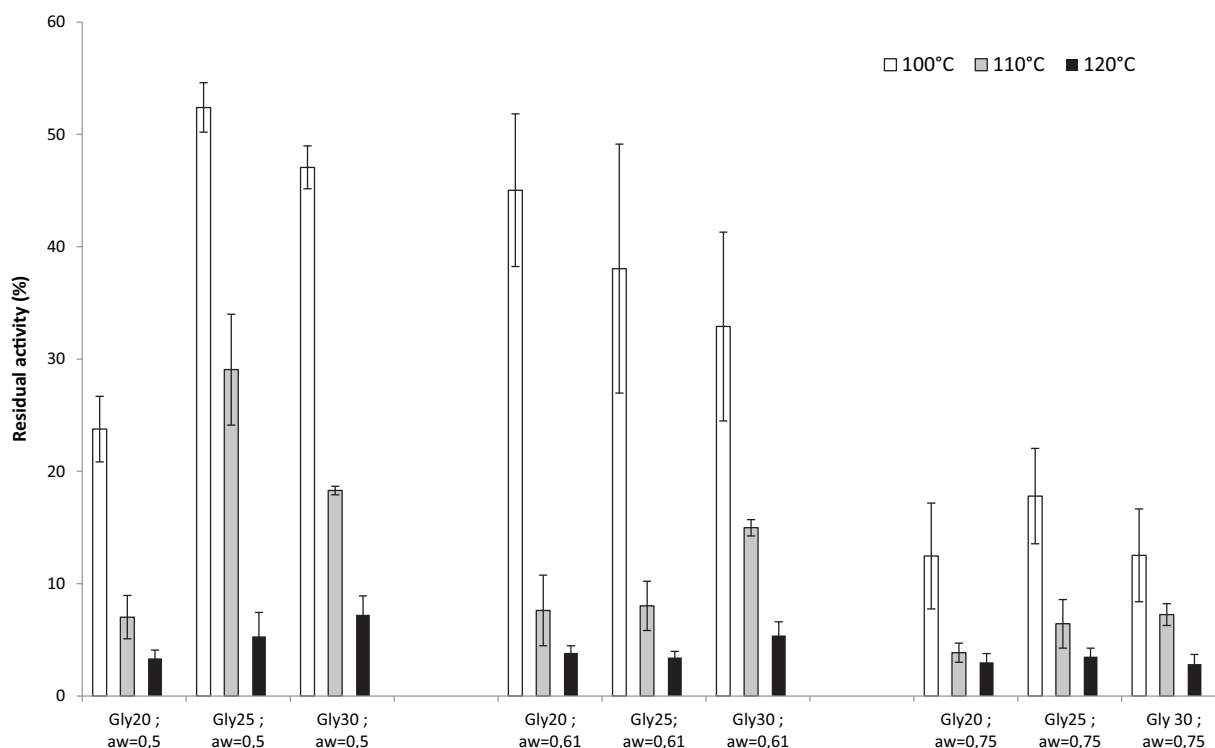
In order to assess only the contribution of corn starch, the main component of corn flour, corn starch-based materials plasticized with water and glycerol and also incorporating 1% (w/w) lysozyme were also prepared by extrusion. Residual activities of lysozyme following extrusion at 83, 93 or 103 °C of TPS (69% w/w)–glycerol (30% w/w)–lysozyme (1% w/w) mixtures with varying initial moisture contents are presented in Fig. 4. The residual activity of lysozyme following extrusion of 30% glycerol–70% corn flour or corn starch formulations previously equilibrated in a 50% RH at 23 °C atmosphere are presented in Table 3. Residual activity of lysozyme following extrusion of corn starch-based blends was always lower than following extrusion of corn flour-based blends.

This suggests that lysozyme embedded in corn flour was more stable than when embedded in corn starch. This increased stability of lysozyme might thus be due to specific interactions with components only present in corn flour. However, since the extruders employed in both cases and processing parameters such as viscosity of the mixture along extrusion zones differed, further studies should be performed to determine the respective contributions of other corn flour constituents than starch, such as zein, fat. . . This could be assessed by following a deformation approach: for instance, native corn flour could be replaced by defatted or deproteinized corn flour in formulations and residual lysozyme activity assayed following their extrusion under similar conditions. When TPS formulation was stored at 23 °C in a 61% or 75% RH atmosphere before extrusion at 93 °C, the residual activity of lysozyme (less than 8%) was significantly lower than when it was stored in a 50% RH atmosphere (18.3 ± 1.4%). As previously observed with corn flour-based formulations, the increase of moisture content favored inactivation of lysozyme during extrusion. However, this trend was not observed following extrusion at 83 °C: the residual activities were similar whatever the initial moisture content of the TPS–glycerol–lysozyme formulation. As foreseen, the increase of extrusion temperature from 83 to 103 °C resulted in an increased inactivation of lysozyme which always had a less than 8% residual activity following extrusion at 103 °C.

As previously stated for corn flour-based formulations, inactivation of lysozyme during TPS formulations extrusion might both result from its thermal denaturation in the presence of water as well as from surface denaturation phenomena enhanced by shear stress.

#### 3.5. Influence of thermoforming temperature and of moisture and glycerol contents in TPS-based formulations containing lysozyme on its stability

After thermoforming for 1 min at 100, 110 or 120 °C of TPS formulations containing 20%, 25% or 30% (w/w) glycerol, various moisture contents and 1% (w/w) lysozyme (Table 1), residual enzymatic activity of lysozyme was assayed (Fig. 5). Moisture content before thermoforming was varied by storing TPS



**Fig. 5.** Effect of thermoforming temperature (100, 110, 120 °C for 1 min), glycerol (20%, 25%, 30% (w/w)) and moisture\* content on the residual activity\*\* of lysozyme incorporated in TPS-based films. \*TPS-based formulations were stored at 23 °C for 72 h before thermoforming in a 47%, 65% or 72% RH atmosphere to obtain formulations with various initial moisture contents. Their water activity ( $a_w$ ) values measured at 25 °C before extrusion were 0.5, 0.61, and 0.75, respectively. \*\*To allow the assay of its residual enzymatic activity, lysozyme was extracted from corn starch-based materials following hydrolysis of corn starch by an amylolytic enzymes preparation as described in Section 2.

formulations for 72 h at 25 °C in a 50%, 61% or 75% RH atmosphere. As expected, whatever the formulation, inactivation of lysozyme increased with thermoforming temperature. The increased inactivation of lysozyme when increasing moisture content of TPS formulations was another general trend. However, this trend was less marked when thermoforming was performed at 120 °C likely because lysozyme inactivation was rapid at this temperature (less than 8% residual activity whatever the formulation). Unlike after extrusion of corn flour-based formulations, increasing glycerol content in TPS formulations did not increase its stability. Residual activity of lysozyme following thermoforming at 100 °C for 2 min of a 70% (w/w) TPS–30% (w/w) glycerol formulation was always higher than following its extrusion at 103 °C (Table 3): residual activity of this formulation previously equilibrated at 25 °C in a 50% RH atmosphere was  $47.1 \pm 1.9\%$  following thermoforming against only  $2.9 \pm 0.1\%$  following extrusion. This suggests that mixing and shear stress associated with extrusion likely also significantly contributed to the inactivation of lysozyme during extrusion (in addition to thermal inactivation enhanced by free water). This would be consistent with Colombié *et al.* [27] observations indicating that mechanical stresses, such as shear forces generated during stirring induce lysozyme inactivation.

#### 4. Conclusion

Lysozyme stability during extrusion with corn flour depended both on formulation parameters such as moisture and glycerol contents and on process parameters such as temperature. Increasing glycerol content in the formulation from 25% (w/w) to 30% (w/w) significantly increased the stability of lysozyme during extrusion at 90 and 100 °C, while increasing moisture content from 14.5% (w/w) to 28.5% (w/w) had the opposite effect. Corn flour-based granules containing active lysozyme could be obtained by

extrusion only when moisture content was limited by storing corn flour in a 50% RH atmosphere at 23 °C and if a 30% (w/w) glycerol concentration was added in the formulation. Glycerol stabilizing effect during extrusion might result from various mechanisms: (i) this plasticizer might reduce the viscosity of the blends during extrusion and thus shear stress, (ii) water, glycerol, and starch form a ternary system in which all these components can be linked by hydrogen bonds. Moreover, water and glycerol can compete for the same binding sites on starch: the respective stoichiometries of water and glycerol could thus explain these apparently contradictory observations. After having addressed the bottleneck of the preparation of active biomaterials by thermomechanical processing, future studies will aim at checking the efficiency of such materials to control contamination by food-borne pathogenic or food-spoiling microorganisms sensitive to lysozyme. Similar studies on the parameters governing stability of enzymes during extrusion should also be performed with other hydrolytic enzymes increasingly used to hydrolyse biopolymers during extrusion of lignocellulosic biomass.

#### Industrial relevance text

The cost of biomaterials on one hand, and the cost and the limited stability of natural food preservatives on the other hand are frequently reported bottlenecks to the launching of active antimicrobial biomaterials. Therefore, the possibility to incorporate lysozyme in corn flour—rather than in corn starch-based materials prepared by extrusion rather than by solvent casting was investigated. Interestingly, a correct choice of glycerol and moisture proportions in corn flour-based formulation allowed to obtain granules retaining more than  $60 \pm 7\%$  of initial lysozyme activity following extrusion at 90 °C.

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