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Properties of lysozyme/*Arthrospira platensis* (*Spirulina*) protein complexes for antimicrobial edible food packaging

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A B S T R A C T

The application of algal proteins to produce active materials is an innovative strategy to explore new packaging for food preservation. In comparison with Na-caseinate as a model film-forming protein, the properties of *Arthrospira platensis* protein isolate (API) complexed with lysozyme (antimicrobial protein) were determined in order to develop edible antimicrobial films. For different pH values (3, 7, or 10), the gradual addition of API (0–4 g/L) induced an increase of turbidity at a constant lysozyme concentration (0.714 g/L). These results were interpreted in terms of API/lysozyme complexes' formation accompanied by a change in their electrophoretic mobility (ζ-potential). This complex formation was shown to considerably decrease the lysozyme antimicrobial activity probably due to a reduction of the enzyme mobility. While increasing the Na-caseinate concentration above the turbidity peaks resulted in aggregates dissociation followed by a recovery of a part of lysozyme antimicrobial activity, the aggregates formed with API were not dissociated. The formed complexes were then applied to make edible antimicrobial films able to control the lysozyme release. A slower lysozyme release was observed at pH 7 because of the attractive interactions between cationic lysozyme and anionic API or Na-caseinate at this pH. Taken together, these results should help to find new applications of algal proteins especially in the field of active food packaging.

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1. Introduction

Microalgal biomass is a potential source of biomolecules, such as β-carotene, polysaccharides, proteins, minerals, and other essential nutrients, which could potentially be exploited as functional food ingredients. The large-scale production of microalgae first occurred during World War II in an effort to drive inexpensive source of proteins in order to supersede those of meat that were difficult to get. By 2010/2011, the global production of microalgal biomass attained 17,000 tons/year [1]. Microalgae are rich in proteins: *Spirulina maxima*: 45%, *Diacronema vlkianum*: 38%, *Isochrysis galbana*: 40% [2], *Haematococcus pluvialis*: 52%, *Chlorella vulgaris*: 51%, and *Porphyridium cruentum*: 58% [3]. The quality and the quantity of microalgal proteins depend on the effectiveness of the cell disruption methods, and also on the structural morphology of each microalgal cell wall [3].

The consumer demands for more natural foods and the desire to protect the environment are the main factors that favored the development of new packaging materials. Different biopolymers, such as polysaccharides, proteins and their blends, were cited in the literature to produce active packaging materials [4,5]. During the last decade, research concerning edible films and coatings as carriers of antimicrobial agents has increased. Several biopreservatives such as organic acids, antimicrobial enzymes, bacteriocins, some plant extracts, and essential oils were integrated in film formulation [4,6–9]. The use of edible films and coatings containing antimicrobials has demonstrated to be a useful tool to protect foodstuff against spoilage flora and to decrease the risk of pathogen growth [5]. Lysozyme (EC 3.2.1.17) is a food grade antimicrobial enzyme with bacteriolytic activity, particularly against Gram-positive bacteria, and efficient in controlling the growth of a great number of food pathogens [10]. Adding EDTA to lysozyme has been shown to improve its antimicrobial activity against some Gram-negative bacteria [4]. Lysozyme is one of the mainly used antimicrobial enzymes incorporated into packaging materials [4,9,11,12].

Antimicrobial films made of algal derivatives have been mainly produced from alginate [8] and carrageenan [7]. To the best of our knowledge, this article proposes the first edible antimicrobial film made with microalgal proteins. In comparison with Na-caseinate, as a largely studied protein with well-known film-forming properties, we studied the complexation of *Arthrospira platensis* proteins with lysozyme. The rationale for the formation of complexes between lysozyme and either sodium caseinate, or *A. platensis* protein isolate prepared by precipitation at pH 3 of *A. platensis* compounds soluble in water at pH 10 is based on the fact that while pI of lysozyme (11.35) was basic, the pIs

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of sodium caseinate and *A. platensis* proteins were 4.6 and close to 3, respectively. This means thus that at pH values close to neutrality lysozyme and sodium caseinate or *A. platensis* proteins had opposite net charges favoring thus the formation of complexes by electrostatic interactions. This justified the choice of acidic proteins to form complexes with lysozyme which is a basic protein. Then, the formed complexes were used to prepare antimicrobial films for food contact packaging applications and the effect of pH on the lysozyme release was also studied.

2. Materials and methods

2.1. Materials

A. platensis powder (89.27 ± 0.16 g/100 g dry matter) was purchased from Bioalgal Society (Mahdia, Tunisia). *A. platensis* protein isolate (API) was extracted from algal powder by dissolution in imidazole/acetate buffer (5 mmol/L, pH 10). After centrifugation for 30 min at 25 °C and 10,000 ×g, the supernatant containing soluble proteins was collected (supernatant A). The pellet was dissolved again in the same buffer and centrifuged under the same conditions and the supernatant was collected (supernatant B). Both supernatants A and B were mixed and adjusted to pH 3 with 0.1 mol/L HCl. The precipitated proteins were then collected by centrifugation (10,000 ×g, 30 min, 25 °C) and dried under a laminar flow hood overnight at 25 °C. The protein isolate residual moisture content after drying was 6.39 ± 0.24 g/100 g of API. The other components were: proteins (69.62 ± 0.75 g/100 g of dry matter), ash (17.79 ± 0.44 g/100 g of dry matter), carbohydrates (8.90 ± 0.88 g/100 g of dry matter), and fats (3.70 ± 0.35 g/100 g of dry matter). All these analyses were determined in triplicate according to AOAC International [13].

Hen egg white lysozyme, in lyophilized form with a specific activity of 70,000 specific units/mg and an isoelectric point (pI) of 11.35, the *Micrococcus lysodeikticus* cells used to measure lysozyme enzymatic activity, analytical grade imidazole ($C_3H_4N_2$), acetic acid, sodium hydroxide (NaOH), and hydrochloric acid (HCl) were purchased from Sigma–Aldrich Chimie (St Quentin Fallavier, France). Deionized water was used for the preparation of all solutions. Sodium caseinate was purchased from Acros Organics (Geel, Belgium).

2.2. Lysozyme/biopolymers complex formation and turbidity measurement

Biopolymer mixtures containing lysozyme (0.714 g/L = 5×0^7 specific specific units/L) and biopolymers: API and Na-caseinate (0–4 g/L) were prepared by mixing different ratios of the stock solutions with imidazole/acetate buffer (5 mmol/L) at different pHs (3, 7, or 10). The turbidity was measured using an UV/Vis spectrophotometer (Jenway 3705, Staffordshire, UK) at 600 nm and 25 °C against pure lysozyme solutions (without API or Na-caseinate).

2.3. Zeta potential measurement

The electrical charge (ζ-potential) of the formed lysozyme/biopolymers (API or Na-caseinate) complexes formed at various biopolymers concentrations and at different pHs (3, 7, or 10) was determined using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). The ζ-potential is determined by measuring the direction and velocity of complexes' movement in the applied electric field. The samples were diluted 10 times with imidazole/acetate buffer adjusted to the suitable pH value. The mean ζ-potential values (± standard deviation) were obtained from the instrument.

2.4. Enzymatic activity of complexed lysozyme

Lysozyme enzymatic activity was assayed by monitoring the absorbance reduction at 450 nm ($OD_{450\ nm}$) due to the lysis of *M. lysodeikticus* cells by lysozyme at 25 °C. Briefly, in a 1 cm cuvette, 2.9 mL of the *M. lysodeikticus* suspension ($OD_{450\ nm} = 1$) in 5 mmol/L imidazole/acetate buffer adjusted to the appropriate pH and 0.1 mL of the enzyme solution (prepared at the same pH) were mixed quickly, and the reduction in the absorbance was recorded using a Jenway 3705 UV/Vis spectrophotometer until reaching a plateau. Lysozyme activity was calculated from the slope of the initial linear portion of absorbance vs. time curve. The hydrolytic activity of the lysozyme solution can be calculated using formula (1) below (one unit of lysozyme activity corresponds to the quantity of active enzyme inducing a 0.001 change in absorbance in 1 min under the assay conditions):

$$\text{Activity (U/mL)} = \frac{S}{0.001 \times V} \tag{1}$$

where S is the slope of the initial linear portion of absorbance vs. time curve and V is the volume of lysozyme solution (0.1 mL).

2.5. Antimicrobial film preparation and characterization

Film-forming aqueous solution was prepared to contain 4.2 g of API or Na-caseinate, sorbitol as plasticizer (0.42 g/g of protein), and lysozyme as an antimicrobial agent (4.285 g/L = 3×10^8 specific units/L). API or Na-caseinate was dissolved in imidazole/acetate buffer (5 mmol/L, pH 10) at 25 °C for 4 h under magnetic stirring. Afterwards, sorbitol and lysozyme were incorporated under the same conditions while stirring overnight. Each film was prepared by weighing and spreading 20 g of film-forming solution evenly over a plastic Petri dish (90 mm diameter). Films were formed by drying overnight at 25 °C under laminar hood and dry films were peeled intact from the casting surface. The final thickness of films was measured at 10 random positions with a Käfer micrometer (Erichsen, Rueil-Malmaison, France).

The film color was measured using a Minolta Chromameter CR-200. Absolute measurements are displayed as Lab tristimulus values (L, a^*, b^* color space). L (from black to white) is the lightness variable, a^* (from red to green) and b^* (from yellow to blue) are the chromatic coordinates. To determine L, a^*, and b^*, three measurements were performed at ten random positions by putting the film on a white reference surface.

A Texture Analyzer (Stable Micro Systems, Godalming, UK) controlled with the texture Exponent 32 software was used to measure elongation at break (EB (%)), tensile strength (MPa), and Young's modulus (Y (Pa)) on 30 mm × 40 mm film samples. Films (stored at 52% RH and 25 °C for 3 days) were uniaxially stretched at a constant rate of 1 mm/s. The elongation at break is the maximum change in film length before breaking, expressed as a percentage. Tensile strength is the maximum film resistance before breaking. Young's modulus was calculated from the slope of the initial linear portion of the force–deformation curve, as follows:

$$Y = (\text{Curve slope}/\text{Film section}) \times \text{Initial film length} \tag{2}$$

$$\text{The film section was} : \text{width} \times \text{thickness} \tag{3}$$

Isotherms were obtained by plotting the moisture content of the samples in equilibrium versus the water activity (a_w) according to the procedure described by Mathlouthi [14]. The small film pieces (30 mm × 40 mm) placed in pre-weighed cups were equilibrated in hermetically sealed flasks containing different saturated salt solutions of known equilibrium relative humidity at 25 °C: LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.52$), NaCl ($a_w = 0.75$), KCl ($a_w = 0.86$), $BaCl_2$ ($a_w = 0.90$), and K_2SO_4 ($a_w = 0.97$). After the samples had reached equilibrium, the film pieces were precisely weighed and the moisture content calculated. The dry matter was measured by the oven-drying method at 105 °C for 24 h. The amount of sorbed water was expressed as g of water/g of dry film.

The amount of lysozyme released from the films to the aqueous solution in which they were immersed was measured according to the

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