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Interactions of tetracyclines with ovalbumin, the main allergen protein from egg white: Spectroscopic and electrophoretic studies

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ABSTRACT

The interactions of tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) with ovalbumin (OVA), the main allergen protein of egg white, were investigated by molecular spectroscopy and electrophoresis at three pH conditions (1.5, 4.6 and 7.4). Molecular and synchronous fluorescence, UV-vis spectroscopy, electrophoresis and ^{1}H NMR were used to study the interaction process. Tetracyclines interact with ovalbumin fluorescence by a static quenching mechanism with non-fluorescent complex formation changing the native protein structure. The binding constant (K_b) ranged from 2.11×10^4 to 58.4×10^4 L mol^{-1}, and corresponding thermodynamic parameters were measured at different temperatures and pH values. The binding process was spontaneous ($\Delta G < 0$), and the magnitude of the interaction increased in the following order: TC < CTC < OTC. Hydrogen, electrostatic, and Van der Waals forces played a major role in stabilizing the complex. The distances between the donor (protein) and receptors (TC, OTC and CTC) were determined by FRET and varied of 2.95–3.52 nm. Fe(III) and Zn(II) ions increase the affinities of TC and CTC for OVA, while OTC did not suffer a significant influence by the competitor metallic species evaluated.

1. Introduction

There are about 40 different proteins in egg white, which are source of amino acids and nitrogen that are required for growth, maintenance, and the general well-being of humans [1]. However, the egg is one of the foods whose allergenicity is most altered by cooking or processing, and egg whites contain proteins with considerably higher allergenic potential [2]. Ovalbumin (OVA) is the major protein from egg white, representing 54% (m/m) of the total protein content, which is comprised of a complex mixture of proteins such as ovotransferrin, ovomucoid, and lysozyme [3]. The chicken egg is one of the most consumed foods in the world because it is inexpensive and an ingredient with a high nutritional value [4]. The industrial importance of ovalbumin is related to its functional properties, in this case its gelation, foaming, and emulsifying potential, among others, which are useful in preparing a variety of food types [5]. Moreover, ovalbumin is used in immunological studies as a carrier protein in vaccines and a model protein in egg sensitivity (allergy) tests [6].

Tetracycline antibiotics are often used as feed additives for food-producing animals because they are antimicrobial agents that exhibit activity against a large range of Gram (−) and Gram (+) bacteria. Due to their broad activity spectrum, low toxicity, and low cost, tetracyclines are widely used as veterinary drugs for the prevention and treatment of farm animals as well as used as additives to promote animal growth [7]. Among the most used tetracyclines for livestock intended for human consumption are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) [8].

The continuous and widespread use of tetracyclines in food-producing animals may result in the presence of their residues in food derivatives. Human exposure to these residues may cause adverse effects from allergic reactions to cancer. Furthermore, exposure to tetracycline residues can produce resistant microorganisms and debilitate the therapeutic action of these drugs [9].

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There are few studies related to the interaction between organic and inorganic species with OVA when compared to studies with human serum albumin (HSA) and bovine serum albumin (BSA). In one such study, Lu et al. studied the interaction between OVA and quercetin using spectroscopic techniques and molecular modelling simulations under physiological conditions [10]. The results suggested that the binding constant value was on the order of 10^4 L mol^{-1} and the main forces involved in the interaction were hydrophobic and hydrogen bonding.

Wang et al. evaluated the interaction of alkaloids present in coffee (caffeine, theophylline, and diprophylline) with OVA using spectroscopic techniques in physiological conditions [11]. The results showed that binding constant values were on the order of 10^4 L mol^{-1}, the distance between the protein and alkaloids varied from 2.20 to 2.25 nm and the main forces involved in the interaction were Van der Waals and hydrogen bonds. Shen et al. evaluated the interaction between OVA and lysozymes with green tea polyphenols simulating gastrointestinal digestion [12]. Additionally, Ognjenovi et al. conducted studies about the interaction between epigallo catechin-3-gallate and OVA. In both studies, the interaction results indicate binding constant values of approximately 10^4 L mol^{-1} [13].

The majority of studies evaluating interactions with OVA are associated with compounds present in foods. However, considering the inadequate practices of veterinary medicine use, the ovalbumin (food protein) could be an indirect contaminate to the consumer since it is widely distributed in different forms. In this context, there is no description of interaction studies of this food protein with veterinary drugs and investigations into the manner by which this process can toxicologically change these species. Thus, the objective of this work was to assess the interactions between OVA and tetracyclines (TC, OTC and CTC) to investigate the most important aspects governing this process *in vitro* at different pH values using spectroscopic techniques and electrophoresis.

2. Materials and methods

2.1. Chemicals and solutions

The protein ovalbumin (*albumin from chicken egg white*) and the antibiotics tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) employed in the interaction studies were purchased from the Sigma-Aldrich Chemical Company (USA). The ovalbumin stock solution (6 μM) was prepared in a 100 mM solution of HCl (pH adjusted to 1.5) containing 100 mM NaCl, and then, the working solutions were prepared by appropriate dilution. Similarly, stock protein solutions were prepared in 50 mM acetic acid/acetate buffer (pH = 4.6) and 50 mM Tris buffer (pH = 7.4), with both containing 100 mM NaCl. The stock ovalbumin *in natura* (without purification) solutions were prepared from solubilizing the egg white aliquot directly in 50 mM Tris buffer (pH = 7.4) containing NaCl. The ovalbumin concentration (commercial and *in natura* form) was determined by the application of Beer's law based on the absorbance signal at 280 nm and molar extinction coefficient (30957 M^{-1} cm^{-1}) after appropriate dilution of the solution [14]. Tetracycline (TC, OTC and CTC) stock solutions were prepared by directly dissolving the solid in ultrapure water at 1.0 mM, and they were stored in amber bottles at 4 °C.

2.2. Fluorescence spectroscopy

Molecular fluorescence measurements were performed on Shimadzu spectrofluorimeter (model 5301PC, Japan) using quartz cuvettes with a 10 mm optical path, height of 45 mm, width of 12.5 mm and depth of 12.5 mm with four polished windows and 3.5 mL of chamber volume. The OVA (2.0 μM) fluorescence emission spectra in the absence and presence of tetracyclines (2–80 μM) were recorded from 250 to 450 nm employing λ_{ex} = 280 nm. The excitation and emission slits were 3 and 5 nm, respectively. All fluorescence assays were carried out in the following three different pH conditions: 1.5, 4.6 and 7.4.

Synchronous fluorescence spectra of the OVA in the absence and presence of different amounts of tetracyclines were obtained by variation in simultaneous excitation and emission monochromators. The difference in the excitation wavelength ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$) was individually fixed at $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm, and thus, the spectrum shows only the spectroscopic behaviour of tyrosine and tryptophan residues, respectively [15].

To assess the protein-ligand interaction and to calculate the critical distance at which the energy transfer occurs from tryptophan residues of OVA (2 μM) to tetracyclines (TC, OTC and CTC all at 2 μM), Förster resonance energy transfer (FRET) studies were performed [16].

2.3. Molecular absorption spectroscopy (UV-vis)

The molecular absorption measurements were performed in a Micronal scanning spectrophotometer (AJX-6100PC model, Brazil) with a double-beam equipped with quartz cuvettes having a 10 mm optical path, height of 45 mm, width of 12.5 mm and depth of 12.5 mm with two polished windows and 3.5 mL of chamber volume. The absorption spectra of complexes of ovalbumin and tetracyclines were recorded in the range of 220–450 nm.

2.4. Polyacrylamide gel electrophoresis (SDS-PAGE and native-PAGE)

The electrophoretic assays (SDS-PAGE and native-PAGE) were performed employing 10% (m/m) polyacrylamide gel. The pH values of the samples were fixed at 7.4, while the concentrations of OVA and the tetracyclines were 18 μM and 9 mM, respectively. Samples were analyzed under reducing and non-reducing conditions at a constant voltage (85 V) using the Mini Protean® system (Tetra Cell, Bio Rad). In the SDS-PAGE, the reducing agent 2-mercaptoethanol (5%, v/v) and sodium dodecyl sulphate (SDS, 2%, m/v) were added into the system. Gel without denaturing (native-PAGE) was performed in the absence of SDS and reducing agent. Protein molecular weight markers (Jena Bioscience) were employed in the experiments.

2.5. Protein–ligand interactions by ^{1}H NMR

In this study, the interactions between the TC and OVA were evaluated by profiling the ^{1}H NMR spectrum and observing variations in the chemical shifts (δ, ppm) of TC in the absence and presence of protein. For this purpose, we used 1.4 mg of OVA and 1 mM tetracycline in 600 μL of phosphate buffer (10 mM, pH = 7.4) with the addition of 10 μL of sodium trimethylsilylpropionate (TMSP) at 2.50 mM (internal standard). All solutions were prepared with deuterated water (Tedia, D_2O, 99.9%). A Bruker 400 NMR spectrometer (B_0 = 9.4 T) operating at 400.35 MHz for ^{1}H and equipped with an indirect detection probe of 5 mm was used.

2.6. Statistical analysis

All experiments were performed in triplicate (n = 3) to ensure accuracy and reliability of the results. In the quantitative evaluations, the results were presented considering the mean value and the respective standard deviation (SD) of three independent experiments. The linear regression analyses for Eqs. (1)–(3) were