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Quillaja bark saponin effects on *Kluyveromyces lactis* β-galactosidase activity and structure

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ABSTRACT

Saponins are known for their bioactive and surfactant properties, showing applicability to the food, cosmetic and pharmaceutical industries. This work evaluated the saponins effects on *Kluyveromyces lactis* β-galactosidase activity and correlated these changes to the protein structure. Enzyme kinetic was evaluated by catalytic assay, protein structure was studied by circular dichroism and fluorescence, and isothermal titration calorimetry was used to evaluate the interactions forces. *In vitro* enzymatic activity assays indicated an increase in the protein activity due to the saponin–protein interaction. Circular dichroism shows that saponin changes the β-galactosidase secondary structure, favoring its protein-substrate interaction. Besides, changes in protein microenvironment due to the presence of saponin was observed by fluorescence spectroscopy. Isothermal titration calorimetry analyses suggested that saponins increased the affinity of β-galactosidase with the artificial substrate *o*-nitrophenyl-β-galactoside. The increase in the enzyme activity by saponins, demonstrated here, is important to new products development in food, cosmetic, and pharmaceutical industries.

1. Introduction

Saponins are a group of natural glycosides, which are widely distributed in plants and some marine animals (Dyck, Gerbaux, & Flammang, 2010; Sparg, Light, & Staden, 2004; Vincken, Heng, Groot, & Gruppen, 2007). These compounds are formed from a hydrophobic aglycone, denominated sapogenin, linked to one or more hydrophilic sugar moieties through an ether or ester glycosidic linkage, at one or two glycosylation sites (Güçlü-Üstündağ & Mazza, 2007). One of the most common sources of saponins employed in the food, cosmetics, and pharmaceutical industries is the Quillaja bark extract, obtained from the tree *Quillaja saponaria* Molina (Nord & Kenne, 1999; San Martín & Briones, 1999). Quillaja saponins are formed predominantly by triterpene bisdesmosides that possess sugar chains consisting of glucose, galactose, xylose, rhamnose, arabinose, and glucuronic acid, substituted at the C-3 and C-28 positions of the triterpene aglycone (Kezwon & Wojciechowski, 2014).

For many years, saponins were predominantly studied for their detergent properties (Güçlü-Üstündağ & Mazza, 2007). Today, the interest in saponins is primarily due to their bioactive properties. Studies demonstrate that the compounds present immunostimulatory, hypocholesterolemic, antitumor, anti-inflammatory, antibacterial, antiviral, antifungal, and antiparasitic activities (El Barky, Hussein, Alm-Eldeen, Hafez, & Mohamed, 2016; Thompson, 1993). Regarding all these properties, Quillaja extract has great potential applications. For commercial purposes, *e.g.*, the extracts are usually mixed with other compounds, such as proteins (Kezwon & Wojciechowski, 2014). The interaction between saponins and proteins can modify the properties of the protein and the solution. In this context, there are found some studies that evaluate their interaction. Potter, Jimenez-Flores, Pollack, Lone, and Berber-Jimenez (1993) evaluated the interaction quillaja saponin-caseins, and it was concluded that their interaction resulted in high molecular weight complexes that presented positive effects on animal blood lipids. The interactions between different saponins and salt soluble proteins from walleye pollack meat were also evaluated by Tanaka, Fang-I, Ishizaki, and Taguchi (1995) and it was concluded that different saponins could modify the proteins aggregation properties. More recently, Kezwon and Wojciechowski (2014) evaluated the effect of β-lactoglobulin and lysozyme on the surface tension of saponins. The different proteins interact with saponins by electrostatic and hydrophobic forces, causing changes in their surface tension. It was also observed that the interaction depends on the specific sites of the

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proteins with sugar or carboxylic moieties of the saponin molecules. Böttcher, Scampicchio, and Drusch (2016) also evaluated the interactions between saponins and β-lactoglobulin. It was suggested that they could interact probably through hydrogen bonds and/or hydrophobic interactions, and changes in the foam properties of the protein were observed. About the effects of saponins on enzymes, it is known that the compound can inhibit the activity of some digestive enzymes, such as lipase, amylase or glucosidase (Birari & Bhutani, 2007; Ercan & El, 2016). As can be observed, soponin promotes important changes in the properties and activity of several proteins, and its knowledge is of great relevance both for the development of new products as well as for the understanding of its physiological aspects.

β-Galactosidase is one of the most important biotechnological enzymes used in the food industry, owing to its ability to hydrolyze the lactose present in milk, which helps to prevent the effects of lactose maldigestion (Gekas & Lopez-Leiva, 1985). The enzyme is a glycoside hydrolase enzyme that catalyzes the hydrolysis of glycosidic bonds, producing monosaccharides from β-galactosides. β-Galactosidase can be utilized in the food industry in the production of lactose-free dairy products, or it can be consumed by the population in pill form when milk products are ingested. In both instances, the reaction occurs in the presence of several other components. It is known that various components can modify the β-galactosidase structure and affect some of the enzyme functional parameters such as V_{max}, K_m, K_{cat}, as well as its catalytic activity (Illanes, Altamirano, & Cartagena, 1994). Some of the substances known to decrease the enzyme activity are silver and copper ions (Wutor, Togo, & Pletschke, 2007), tetracycline hydrochloride (Gao, Bi, Zuo, Jia, & Tang, 2017), and copper oxide nanoparticles (Rabbani, Jahir, Ahmad, Yusof, & Hasan, 2014). Examples of substances known to enhance β-galactosidase activity are milk proteins (Greenberg & Mahoney, 1984), ions Na^+ and K^+ at specific conditions (Reithel & Kim, 1960) and low concentrations of calcium and ferrous ions (Wutor et al., 2007).

About the saponin and enzymes interactions, it has been shown that they can interact with each other and change their properties. Bouarab, Melton, Peart, Baulcombe, and Osbourn (2002) verified that the fungal saponin-detoxifying enzyme (tomatinase) could hydrolyze antimicrobial saponins, causing a suppression on plants defenses responses and favoring plants infection. Ishaaya and Birk (1965) reported that soybean saponins interact and can inhibit, to a certain extent, enzymes such as cholinesterase, chymotrypsin, trypsin, and papain. Luyen, Dang, Binh, Hai, and Dat (2018) demonstrated that a triterpenoid saponin isolated from *Polyscias fruticosa* leaves strongly inhibited the enzymes α-amylase and α-glucosidase. Additionally, it was observed that the saponin also decreased the postprandial blood glucose level in a mouse model. Studies about the interactions of β-galactosidase with saponins are still scarce. Zhang et al. (2015) evaluated the interaction of *Lactobacillus bulgaricus* β-galactosidase and steroidal saponins. It was reported that the enzyme could glycosylate saponins using lactose as a donor substrate. The results provide a new way to generate compounds with a novel or improved properties. Given the changes caused by saponins on properties, structure, and functionality of different enzymes, further investigations on the topic are required.

Due to the importance of β-galactosidase for the pharmaceutical, chemical, and food industry, the enzyme was chosen as an object of this study. Additionally, in the current scenario, where saponins are being studied for their bioactive properties, that can provide new properties to other products, it is important to evaluate their interactions with different enzymes. Knowing that the interaction of saponins with β-galactosidase has not yet been characterized, this work aims to assess the effect of saponins on the enzyme activity, by monitoring the changes in the enzyme kinetic parameters (V_{max}, K_{cat}, and K_m). Furthermore, these data were correlated with transformations in the enzyme's secondary and tertiary structure upon saponin exposure by using circular dichroism and fluorescence spectroscopy. Finally, the effect of saponins on the interaction of β-galactosidase synthetic substrate was evaluated by isothermal titration calorimetry (ITC).

2. Materials and methods

2.1. Materials

The enzyme β-galactosidase (EC:3.2.1.23) produced by *Kluyveromyces lactis* was provided by Granolab Granotec company (Araucária, Paraná, Brazil). The β-galactosidase activity was described by the manufacturer as 6500 acid lactase units per gram, and its concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as the standard, and the concentration obtained was 113.84 mg mL^{-1} or 0.967 mmol L^{-1}. The enzyme was analyzed by SDS-PAGE previously (data presented in Kayukawa et al., 2018). The results showed that the enzyme, with a molar mass of ~117 KDa, was the predominant protein of the sample. The densitometric analyses of the protein bands in that gel indicated 70% of purity of β-galactosidase.

The reagents *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (> 98% purity; molar mass of 301.25 g mol^{-1}) and 2-nitrophenol (ONP) (98% purity; molar mass of 139.11 g mol^{-1}) were obtained from Sigma–Aldrich (St Louis, Missouri, USA). Concentrated extracted of Quillaja bark saponin (QBS) was obtained from Vetec Sigma-Aldrich (Rio de Janeiro, Brazil). According to the supplier, the concentration of saponin in the extract is < =100% (CAS number: 8047-15-2). A Milli-Q system (Millipore Corp., Billerica, Massachusetts, USA) was used to provide deionized water.

2.2. Methods

2.2.1. Enzyme activity assay

The hydrolytic activity of β-galactosidase was evaluated using the synthetic substrate ONPG. The methodology was adapted from Inchaurrondo, Yautorno, and Voget (1994). The enzyme activity assay was performed with samples constituted by 75 μL of 6.37×10^{-3} μmol L^{-1} β-galactosidase enzyme, 75 μL of 1.5 mmol L^{-1} artificial substrate ONPG and 775 μL of potassium phosphate buffer pH 6.8, 75 mmol L^{-1}. In the case of saponin samples, the amount of buffer used was 700 μL, and the protein and additive quantities were both 75 μL. The QBS extract concentration was 0.05 mg mL^{-1}, chosen according to preliminary enzymatic assays and ITC assays. After preparing the samples, they were promptly conditioned at 37 °C in the thermostatic bath Dubnoff (304/D, Ethik Technology, Vargem Grande Paulista, São Paulo, Brazil). Sodium carbonate solution (75 μL; 2 mol L^{-1}) was added to each sample to stop the reaction in intervals ranging from 1 to 240 min. The absorbance was measured in a UV–Vis spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan) at 420 nm. To quantify the enzymatic product formation, an ONP standard curve was constructed, and the results were expressed in mmol L^{-1} (see Supporting information). The samples enzymatic activity was calculated as follows:

$$Ea = \frac{A.\ V_{reaction}}{\varepsilon.\ V_{enz}.\ t} \quad (1)$$

where *Ea* represents the enzyme catalytic activity in U mL^{-1}. *A* is the absorbance measured (420 nm), $V_{reaction}$ consist of the volumes present in the reaction in mL (buffer solution + ONPG solution + enzyme solution + sodium carbonate solution), ε (4024.66 L mol^{-1} cm^{-1}) is the extinction coefficient of ONP under the experiment and measured at 420 nm, V_{enz} is the volume of the enzyme extract in mL, and *t* is the time in minutes. Each of the β-galactosidase unit (*U*) represents the quantity necessary of the enzyme to release 1 μmol *o*-nitrophenol per minute under the assay conditions.

The kinetic parameters, i.e., the Michaelis–Menten constant (K_m), maximum reaction rate (V_{max}) and turnover number (k_{cat}) of the effects of the saponin on the β-galactosidase and ONPG interaction, were