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In vitro and *in silico* studies of the inhibition activity of anthocyanins against porcine pancreatic α-amylase

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ABSTRACT

The inhibition activities and mechanisms of four anthocyanins including cyanidin-3-glucoside, cyanidin-3,5-glucoside, cyanidin-3-rutinoside, and peonidin-3-glucoside, against porcine pancreatic α-amylase were investigated through *in vitro* and *in silico* studies. The *in vitro* inhibition study demonstrated that the four anthocyanins competitively inhibited porcine pancreatic α-amylase, which was later verified by the *in silico* molecular docking study that showed all the anthocyanins bound exclusively to the active site of porcine pancreatic α-amylase. Cyanidin-3-glucoside was found to have the highest inhibition activity with the K_i value of 0.014 mM, followed by cyanidin-3-rutinoside, cyanidin-3,5-glucoside, and peonidin-3-glucoside with the K_i value of 0.019, 0.020, and 0.045 mM, respectively. Results obtained from the *in silico* study also showed that the four anthocyanins were surrounded by the side chains of the active site of porcine pancreatic α-amylase, among which the side chain of GLU233 was supposed to play a key role in imparting the inhibition activity of anthocyanins.

1. Introduction

The chronic disease diabetes mellitus is a metabolic disease that is characterized by hyperglycaemia. Approximately 90–95% of all diagnosed diabetes are Type II diabetes mellitus, which is previously called non-insulin-dependent diabetes (American Diabetes, 2010). The number of patients with diabetes has sharply risen in both developed and developing countries in last few decades. The United States Centers for Disease Control and Prevention (CDC) reported that in USA the number of people with diagnosed diabetes more than tripled (from 5.6 million to 20.9 million) over the period of 1980 to 2011 (Centers for Disease Control and Prevention, 2013).

The intake of natural antioxidants from fruits and vegetables has been associated with health benefits over the years (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Sui, Bary, & Zhou, 2016; Sun, Bai, Zhang, Liao, & Hu, 2011a). One of the most well-known natural antioxidants found in most fruits and vegetables is anthocyanins. Anthocyanins (from Greek, *anthos* means flower, and *kyanos* means blue) belonging to flavonoids group are naturally occurring pigments that are responsible for the orange, red, violet, and blue colours observed in nature (Sui, Dong, &

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Zhou, 2014; Sui, Yap, & Zhou, 2015; Sun, Bai, Zhang, Liao, & Hu, 2011b). The well-known health benefits of anthocyanins is their antioxidant capacity due to their peculiar chemical structure that can react with reactive oxygen species, such as superoxide, singlet oxygen, peroxide, hydrogen peroxide, and hydroxyl radical (Bueno et al., 2012; Sui & Zhou, 2014; Sun, Cao, Bai, Liao, & Hu, 2010). Beyond antioxidant capacity, recent studies have drawn more attention to their inhibition activity against digestive enzymes (Sui, Zhang, & Zhou, 2015). Matsui et al. (2001) found that morning glory (*Pharbitis nil* cv. Scarlett O'Hara) flowers rich in anthocyanins were effective inhibitors of human salivary α-amylase. Wiese, Gärtner, Rawel, Winterhalter, and Kulling (2009) observed that the activity of porcine pancreatic α-amylase decreased with increasing the concentration of cyanidin-3-glucoside. Tsuda, Horio, Uchida, Aoki, and Osawa (2003) found that high-fat-diet-induced hyperglycaemia, hyperinsulinaemia, and hyperleptinaemia of mice were reduced by feeding mice using diets containing purple corn which was rich in cyanidin-3-glucoside. They further suggested that the use of anthocyanins as a functional food ingredient may have benefits for the prevention of obesity and diabetes.

However, so far, studies on the inhibition activities and mechanisms of anthocyanins against α-amylase have been very limited, not to mention their molecular level interactions with α-amylase. Thus the aim of this study was to investigate the inhibition activity and mechanisms of four anthocyanins against porcine pancreatic α-amylase through *in vitro* inhibition and *in silico* molecular docking studies.

2. Materials and methods

2.1. Materials

Cyanidin-3-glucoside, cyanidin-3-rutinoside, peonidin-3-glucoside standards were purchased from Tokiwa Phytochemical Co., Ltd. (Chiba, Japan). Cyanidin-3,5-diglucoside standard was purchased from ChromaDex (Irvine, CA, USA). Porcine pancreatic α-amylase (type VI-B, from porcine pancreas), corn starch, and 3,5-dinitrosalicylic acid reagent (DNS) were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA).

2.2. Inhibition activity and mechanism study

The inhibition activity of anthocyanins against α-amylase was determined by measuring the reducing power of released oligosaccharide from soluble starch according to the method of Miller (1959) with minor modifications. A series of tests at varying concentrations of both substrate and anthocyanins were conducted to determine inhibition parameters and types. The varying substrate concentrations of 2.5, 5, 10, and 15 mg/mL were prepared by dissolving corn starch in sodium phosphate buffer (SPB, 0.1 M, pH 6.9) followed by gelatinizing the solution at 100 °C for 15 min. Similarly, anthocyanin solution concentrations of 0, 1.5, 3, and 6 mg/mL were prepared by dissolving anthocyanins in SPB. Porcine pancreatic α-amylase was also dissolved in SPB at a fixed concentration of 1 mg/mL. All the SPB was freshly prepared prior to each test and fortified with calcium chloride at 40 mg/L as calcium is an essential cofactor for α-amylase (Morris, Fichtel, & Taylor, 2011). Aliquots of an α-amylase solution of 20 μL and an anthocyanin solution of 20 μL were mixed in a 2 mL Eppendorf tube and incubated at 37 °C for 15 min to allow interactions between α-amylase and anthocyanins. The reaction was initiated by adding 60 μL of starch solution into the mixture and lasted for another 5 min at 37 °C. Afterwards, an aliquot of 100 μL of 3,5-dinitrosalicylic acid (DNS) reagent solution was added followed by heating in boiling water for 15 min to develop colour. The reaction was then stopped by cooling down in ice water. The absorbance of the reaction mixture was read at 540 nm using a spectrophotometer (UVMini 1240, Shimadzu, Kyoto, Japan). To avoid the colouring effect of anthocyanins, the spectrophotometer was blanked respectively for different anthocyanin solutions using samples prepared in the same way but replacing α-amylase and starch solution with SPB. A control sample was similarly prepared by replacing anthocyanin solution with the same amount of SPB.

The type of enzyme inhibition was graphically determined using the Lineweaver–Burk plot followed by calculating the inhibition constant (K_i) through global nonlinear regression of all data sets at once using GraphPad Prism software (Intuitive Software for Science, San Diego, CA, USA). The inhibition activity (IC_{50}) was used to evaluate the effectiveness of an inhibitor. IC_{50} value is defined as the concentration of a test substance required to achieve half maximal inhibition of a given reaction. IC_{50} values for the four anthocyanins against α-amylase were calculated using the Cheng–Prusoff equation (Yung-Chi & Prusoff, 1973) (Eq. (1)).

$$IC_{50} = K_i\left(1 + \frac{[S]}{K_m}\right) \quad (1)$$

where K_i is the inhibition constant. [S] is the concentration of substrate and its value was fixed at 5 mg/mL in the calculation of IC_{50} for each anthocyanin. K_m is the Michaelis–Menten constant; its value can be obtained from Eq. (2) and was 20.97, 3.75, 5.93 and 7.87 mg/mL for cyanidin-3-glucoside, cyanidin-3,5-glucoside, cyanidin-3-rutinoside and peonidin-3-glucoside, respectively.

2.3. In silico molecular docking study

To investigate the interactions between the four anthocyanins and porcine pancreatic α-amylase, an *in silico* molecular docking study, which can predict the structure of a ligand within the constraints of a receptor binding site (Yuriev & Ramsland, 2013), was performed. The three-dimensional structure of porcine pancreatic α-amylase complexed with acarbose (PDB ID:1OSE) was obtained from the online Protein Data Bank (PDB). The complexed acarbose was removed using ChemBio 3D Ultra software (version: 12.0, Cambridge Soft). The molecular docking was implemented using AutoDock Tools (ADT, version: 1.5.6) with the help of AutoDock 4.2 package (Autodock 4.2 and Autogrid 4.2). Although including water molecules into docking may lead to improvement in pose prediction, it has been suggested that such improvement may be system or procedure dependent (Yuriev & Ramsland, 2013). Therefore, water molecule was removed from the crystal structure of porcine