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Inhibition of cyclodextrins on α-galactosidase

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ABSTRACT

This work successfully investigated the effects of different influential factors and hydrophobic cavities of cyclodextrins (CDs) on α-galactosidase (α-Gal) by detecting α-Gal activity. The highest inhibitory concentration of three kinds of CDs (α-, β-, and γ-CD) on α-Gal was 10 mM. Moreover, the highest inhibition of α-Gal was obtained under the following conditions: reaction time of 90 min, temperature of 30 °C, and pH 6.0. Compared with other CDs, β-CD showed more ability to interact with α-Gal due to its appropriate cavity geometric dimensions. From circular dichroism and nuclear magnetic resonance it was observed that β-CD changed the secondary structure of α-Gal and formed a hydrogen bond with this enzyme.

1. Introduction

Cyclodextrins (CDs) are nontoxic macrocyclic oligosaccharides, consisting of (α-1, 4)-linked α-L-glucopyranose units. CDs are very attractive ingredients for making artificial enzymes and other biomimetic materials (Tong, 2001).

The most significant characteristic of CDs is the certain size of stereoscopic chiral cavity with hydrophobic central cavity and hydrophilic outer surface (Del Valle, 2004). According to the number of glucose units, the degrees of polymerization are 6, 7 and 8 for α-, β- and γ-CD, respectively.

CDs have the ability to form inclusion or non-inclusion complexes with different compounds, such as organic molecules, rare gases and inorganic compounds (Hedges, 1998; Szejtli, 1998). This fact allows CDs to slow the release of some target compounds, improving the solubilization and the antioxidant potential of some components as well as masking some negative properties of some products (eg. bad smell). For all the above mentioned reasons, over the last years, people have become more and more interested in the study of CDs and the research regarding the modification of cyclodextrin is nowadays widely used in food, medicine, chemistry, agriculture, environmental protection and other fields.

α-Galactosidase (α-Gal, EC 3.2.1.22) is an enzyme exoglycosidase, and the main role of this enzyme is the enzymolysis of α-galactoside. After this hydrolysis, galactosylation of cyclodextrins by the α-galactosidase occurs, thus forming α-galactosyl-cyclodextrin. This branched CD is easy to dissolve in water, and has several applications. Some previous studies have found that alphathe α-galactopyranosyl-cyclodextrin (α-galactopyranosyl-CD) can be used as a tool in the delivery of some important healthy compounds. For instance, some authors found that α-galactopyranosyl-CD increased significantly the solubility of retinoic acid (RA) in liver cells. And other works showed the inhibitory effect of α-galactopyranosyl-CD on the combination of lactose-carrying styrene homopolymer (PVLA) and liver cancer cells *in vitro*, thus showing the ability of this complex to be used as drug target for liver cells to the carrier (Abe, Kenmoku, Yamaguch, & Hattori, 2002; Connolly, Townsend, Kawaguchi, Bell, & Lee, 1982; Oda, Yanagisawa, Maruyama, Hattori, & Yamanoi, 2008; Seo et al., 2004; Shinoda et al., 1998).

Some previous studies have used the α-Gal from green coffee beans to synthesize the α-galactopyranosyl-cyclodextrin (α-galactopyranosyl-CD), being the enzymatic synthesis the only existing method. However, the yield of the α- galactopyranosyl--CD was low (about 24%) (Hara et al., 1994; Kitahata, Hara, Fujita, Kuwahara, & Koizumi, 1992; Koizumi et al., 1995; Okada, Koizumi, & Kitahata, 1996).

In this line, some research results showed that CDs may change the process of enzymatic reaction and form complexes with special groups of the enzymes, thus activating or inhibiting the enzyme

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activity. Moreover, they also affected both the reaction rate and equilibrium (Hamilton, 2000; Pinotsis, Leonidas, Chrysina, Oikonomakos, & Mavridis, 2003; Thoma & Koshland, 1960).

In previous studies, the interaction between glucoamylase and CDs was studied. It was observed the potential of glucoamylase to induce proteolytic removal on substrate specificity and its subsequent inhibition by β-CD (Monma, Yamamoto, & Kainuma, 1989; Fagerström, 1994). In addition, some other authors, found biomimetic reactions catalyzed by CDs and their derivatives (Breslow & Dong, 1998).

Overall, it has been shown that CDs increased the utility of enzymes in organic synthesis (Harper, Easton, & Lincoln, 2000). For instance, some previous results showed the ability of CDs to limit substrate inhibition and alter substrate selectivity displayed by enzymes (Easton, Harper, Head, Lee, & Lincoln, 2001). And, other authors investigated the inhibition mechanism of β-CD on pullulanase (Yu, Tian, Yang, Xu, & Jin, 2011), thus concluding that CDs and derivatives decreased the activity of this enzyme.

Previous studies mainly focused on the enzymatic properties and activity of α-Gal. However, there are few reports on how CDs affect the microenvironment and secondary structure of α-Gal. And, to the best of our knowledge, no previous studies have been focused on α-Gal inhibition induced by CDs. In the synthesis of α- galactopyranosyl-CD, CDs, α-Gal and other materials were used in one enzyme synthesis system.

Based on the results obtained for other enzymes, CDs have the potential to inhibit α-Gal, thus leading to a low yield in the formation of the α-galactopyranosyl-CD complex. Thus, it is of a paramount importance to investigate what factors are influencing the inhibition of CDs on α-Gal.

Therefore, this work will focus on understanding the inhibition induced by α-, β- and γ-CD on α-Gal. Moreover, the theoretical foundation for reducing the inhibitory effect of CDs on α-Gal and improving the synthesis efficiency of α-galactopyranosyl-CD complex will be established. For this purpose, the activity of α-Gal will be determined by measuring the concentration of the substrate *p*-nitrophenyl-α-D-galactopyranoside (PNGP). Moreover, the α-Gal enzyme activities will be evaluated and compared without addition of CDs and after adding CDs. The best inhibitory effect will be selected by comparing the ability of α-, β- and γ-CD to inhibit the α-Gal enzyme at different conditions (temperature, pH, concentration, time, etc.). For this purpose, circular dichroism and nuclear magnetic resonance techniques will be used.

2. Materials and methods

2.1. Chemical and reagents

α-Galactosidase (α-Gal, EC 3.2.1.22) was extracted from green coffee beans, *p*-Nitrophenyl-α-D-galactopyranoside (PNPG), CDs (α-CD, β-CD and γ-CD) and 3-trimethylsilyl-1-propanesulfonic acid sodium salt (DSS) were purchased from Sigma–Aldrich (Shanghai) Trading Co., Ltd. Deuterium oxide (D_2O) was purchased from Xiya Reagent Co., Ltd. All other regents were commercial preparations of analytical grade.

2.2. α-Galactosidase (α-Gal) activity determination

The activity of α-Gal was determined based on the degree of release of *p*-nitrophenyl-α-D-galactopyranoside (PNPG). In the assay, 0.01 mL of enzyme solution were incubated with 0.02 mL of 9.9 mmol/L PNPG in 0.07 mL potassium phosphate buffer (pH 6.5) at 25 °C for 5 min. The reaction was stopped by adding 0.2 mL of 0.2 mol/L borate buffer (pH 9.8). *p*-nitrophenol released from substrate was measured with a spectrophotometer at 405 nm. One unit of α-Gal activity was defined as the necessary for hydrolyzing 1.0 μmol of PNPG to *p*-nitrophenol and D-galactose per minute (Marraccini et al., 2005; Shen et al., 2008, 2009). The relative residual activity (RRA) of α-Gal was defined as a percentage of the α-Gal induced by CDs relative to that of the enzyme sample without CD. The relative enzyme activity (RRA) was calculated as follows (Eq. (1)):

$$\text{Enzyme activity}(Unit/ml) = \frac{(A_{405nm}\text{sample} - A_{405nm}\text{blank}) \times 0.3 \times \text{df}}{18.5 \times 5 \times 0.01} \quad (1)$$

where df is the enzyme liquid dilution ratio; $A_{405\ nm}$ sample is the absorbance of the samples at 405 nm, and $A_{405\ nm}$ blank is the absorbance of the blank group at 405 nm.

2.3. Circular dichroism spectra

Circular dichroism spectra were scanned at far-UV range (190–250 nm) with J-810 Chiral Detector (Jasco, Japan) in a 0.1 cm quartz cuvette at 25 °C. The concentration of protein for circular dichroism analysis was 1.12 mM. Three scans were averaged to obtain the circular dichroism data which were expressed in terms of molar ellipticity ([θ]), in degree cm^2/dmol. The estimation of secondary structure was performed using the K_2D method (Yang, Wu, & Martinez, 1986), which is based on the linear regression method of Young's algorithm. For this method, the 1.10.00 Windows Secondary Structure Estimation Version was used. The fitting procedure assumed that the secondary structure of the protein to be detected is the same to that found for the 4 kinds of conventional secondary structure of the α- helices, β-folding, turn the corner and random coil of a standard protein. The measured samples were repeated fitting each conventional structure and the percentage of each secondary structure was calculated. This calculation method requires fitting the error in a certain range of reference. At the same time, measured protein and reference protein types were required to be roughly similar.

2.4. Proton nuclear magnetic resonance (^{1}H NMR) analysis

^{1}H NMR spectrum of the sample was tested by Bruker Avance 600 NMR spectrometer. Samples and internal standard DSS were dissolved in D_2O, and detected in the inner diameter of 5 mm NMR tube at a dedicated frequency, the cumulative number was 128 times. And the data processing software was Topspin 3.2 nuclear magnetic database and processing software.

2.5. Statistical analyses

All experiments were conducted in triplicate. All statistical analyses were performed using the software SPSS Version 22 (IBM® SPSS® Statistics, USA). Significant differences between the results were calculated by multiple sample comparison of the means (ANOVA) and the LSD test, with a significance level of $p < 0.05$. The error bars presented on the figures correspond to the standard deviations.

3. Results and discussion

3.1. Effects of concentration of CDs on α-Gal activity

In order to study the effects of CDs on α-Gal from green coffee beans, the relationship between α-Gal activity and the concentration range of CDs was examined. CDs at different concentrations (0, 0.01, 0.1, 1, and 10 mM) were added into the reaction system (pH 6.5) and the reaction was followed for 60 min at 25 °C.