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ORIGINAL ARTICLE

A study on the inhibition mechanism of β-cyclodextrin on pullulanase

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Abstract Effects of β-cyclodextrin on the activity and structure of pullulanase were investigated in this work. Inhibition of β-cyclodextrin on pullulanase depended on concentration and tended to be enhanced with the increase of β-cyclodextrin. The increase of intrinsic fluorescence induced by β-cyclodextrin was observed, which was probably due to the formation of inclusion complexes between aromatic amino acid residues of pullulanase and β-cyclodextrin. The α-helix content in pullulanase decreased from 15 to 9% but the β-sheet content increased from 35 to 43% with the increase of the concentration of β-cyclodextrin from 0 to 10 mM. The ratio variation of α-helix to β-sheet was almost accordance with that of relative residual activity of pullulanase, especially in the presence of high concentration of β-cyclodextrin. These results showed that β-cyclodextrin changed the secondary structure and microenvironment of pullulanase, accordingly leading to the loss of enzymatic activity.

Keywords Pullulanase · β-cyclodextrin · Inhibition · Conformation changes · Secondary structure

Introduction

Cyclodextrins (CDs) are a family of cyclic oligosaccharides consisting of glucopyranose subunits linked by α-(1, 4) glycosidic bonds. Owing to their hydrophobic cavities which can entrap hydrophobic molecules, CDs have been widely served as host molecules or molecular chelating agents in supramolecular chemistry [1, 2]. In many biological reactions, CDs and their derivatives can form inclusion complexes with substrates or functional groups of enzyme including aromatic amino acid residues [3, 4]. Effects of CDs and their derivatives on enzyme reactions have been widely studied in recent years. These researches usually focus on two sections: one is demonstrating how CDs and their derivatives increase the availability of substrates or reduce the inhibition derived from substrates or products [5, 6]; the other is investigating the shift of enzymatic equilibrium when CDs and their derivatives are added into enzyme reactions [7, 8]. CDs and their derivatives could be acted as enzymatic browning inhibitors in fruit juices such as apple juice and banana juice [9–11]. Koralewska et al. reported effects of cyclodextrins derivatives on the catalytic activity of tyrosine phenol-lyase, but CDs interacted with the enzyme in an unknown way to influence its catalytic behavior [12]. Inhibition by CDs of chymotrypsin-catalysed hydrolysis of N-acetyl-*l*-tyrosine ethyl ester and N-succinyl-L-phenylalanine p-nitroanilide was due to steric effects at the substrate rather than direct interaction with the enzyme [13].

Earlier researches have described the inhibition by CDs of α-amylase family. Cyclodextrins could inhibit β-amylases and this inhibition was turned out to be competitive for both plant and microbial β-amylases [14–16]. Hydrolysis of both soluble starch and raw starch by the amyloglucosidases were inhibited by β-cyclodextrin (β-CD) [17, 18], nevertheless it remained unclear whether β-CD interacted with either the active site or the affinity site on this enzyme. Cyclodextrin glycosyltransferases were also inhibited by CDs in mixed type and the interaction with CDs at the binding site might be blocking access to the active site of the enzyme [19, 20]. Pullulanase

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(EC 3.2.1.41) is a well-known starch-debranching enzyme, which specifically cleaves α-(1, 6) glycosidic bonds in amylopectin, pullulan and glycogen. The interaction between pullulanase from *Klebsiella pneumoniae* and CDs has been examined by means of inhibition kinetics. CDs were found to be competitive inhibitors and β-CD showed strong inhibition [21–23]. Previous studies mainly focus on kinetics to reveal inhibition mechanisms of CDs on catalytic activity of pullulanase. However, there are limited reports on how CDs affect the secondary structure and microenvironment of pullulanase. With regard to enzyme molecules, activation and inhibition properties of cyclodextrins were up to their hydrophobic cavities which were function of size or shape recognition for structure and conformation of enzymes. Thus, more detailed information about structural features of pullulanase in the presence of β-CD could contribute to reflect the actual interaction between β-CD and pullulanase. The aim of this study was to evaluate effects of β-CD on pullulanase by exploring structure and microenvironment changes.

Experimental

Pullulanase from *Bacillus acidopullulyticus* was purchased from Genencor Inc. (Wuxi, China). β-CD was purchased from Sigma-Aldrich Trading Co., Ltd (Shanghai, China). All other chemicals and reagents were of reagent grade.

Pullulanase was dialyzed overnight against acetate buffer (20 mM, pH 5.0) to remove a great many saccharides and then concentrated against polyethylene glycol 20,000 at 4 °C. The concentrating enzyme was further purified through HiPrep16/10DEAEFF ion exchange chromatography and HiPrep16/60Sephacryl S-200HR chromatography. All subsequent chromatographic steps were performed by the application of Akta Purifier 10 system (Pharmacia Amersham Biotech, Sweden). The purified pullulanase was analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and was certified a single band.

Pullulanase activity was determined by measuring the amount of enzyme to release reducing sugars during incubation with pullulan. In this assay, the reaction mixture containing 0.5 mL of 1% (w/v) pullulan in 20 mM acetate buffer (pH 5.0) and 0.5 mL of enzyme sample was incubated at 50 °C for 30 min. The reducing sugar released was determined by dinitrosalisyilic acid method (DNS) [24]. One unit of pullulan activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of reducing sugars per minute. A V-1800 spectrophotometer (Mapada, Shanghai, China) was used for all assays.

Relative residual activity (RRA) of pullulanase was defined as a percentage of activity of the pullulanase induced by different concentrations of β-CD relative to that of the enzyme sample without β-CD.

Fluorescence spectra were recorded using an F-7000 fluorescence spectrometer (Hitachi, Japan) at 295 nm (excitation wavelength, slit = 5.0 nm), 200–400 nm (emission wavelength, slit = 5 nm) and 12,000 nm/min of scanning speed.

Circular dichroism spectra were scanned at far-UV range (190–250 nm) with MOS-450 Chiral Detector (Biologic Science Instruments, Grenoble, French) in a 0.1 cm quartz cuvette at 25 °C. The concentration of protein for circular dichroism analysis was 0.2 μM. Five 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 method of K2D (http://www.embl.de/~andrade/k2d.html).

Results and discussion

Effect of β-CD on pullulanase activity

In order to study effects of β-CD on pullulanase from *Bacillus acidopullulyticus*, the relationship between pullulanase activity and the concentration range of β-CD has been examined. Increasing concentrations of β-CD (0, 0.01, 0.1, 1, and 10 mM) have been added into the reaction system containing 0.2 μM pullulanase. As shown in Fig. 1, the inactivation rates of pullulanase were found to increase with increasing amounts of β-CD. Interestingly, slight activation of pullulanase was observed in the presence of 0.01 and 0.1 mM β-CD, but in the presence of more than 1 mM β-CD, the activity of pullulanase quickly decreased and only remained less than 40% induced by 10 mM β-CD. This indicated that the relationship between pullulanase and β-CD was dependent on their concentrations. Similar results have been observed in previous studies and β-CD was found to be strong competitive inhibitors [21–23]. As is well known, the decrease in enzyme activity attributes to the change of enzyme

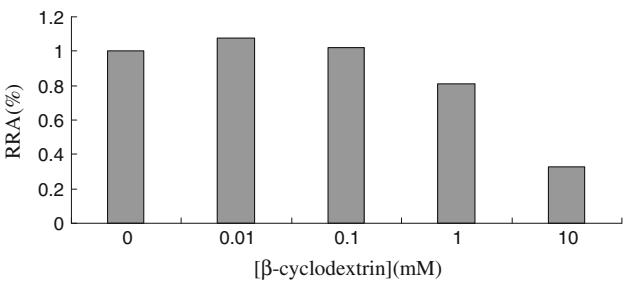

Fig. 1 Effects of the concentration of β-CD on pullulanase activity

structure induced by external factors. Consequently, in order to explore inhibition mechanisms of β-CD on pullulanase, variations of pullulanase microenvironment and secondary structure were detected by fluorescence spectra and circular dichroism spectra.

Analysis of intrinsic fluorescence induced by β-CD

Fluorescence spectroscopy is an effective technique to follow tertiary structure transitions in enzymes. Generally, the intrinsic fluorescence of protein derives from aromatic amino acids such as tryptophan, phenylalanine and tyrosine. Actually, in many proteins or peptides tryptophan mainly contributes intrinsic fluorescence on account of low phenylalanine yield and easy fluorescence quenching of tyrosine [25]. Moreover, the intrinsic fluorescence of tryptophan is particularly sensitive to the polarity of microenvironment [26]. Therefore, tryptophan residues are usually used as endogenous probes to reveal the change of tertiary structure and microenvironment of enzymes. As shown in Fig. 2, the fluorescence spectrum of native pullulanase excited at 295 nm had a maximum fluorescence emission at 340 nm. The fluorescence intensity gradually increased with the increasing of β-CD. This indicated that β-CD likely formed inclusion complexes with tryptophan residues to protect the fluorescing singlet state; on the other hand, the cyclodextrin cavity afforded an apolar surrounding for tryptophan to enhanced fluorescence quantum efficiencies. It was identical that CDs could result in significant fluorescence enhancement when they formed inclusion complexes with analyte chromophore [27]. However, β-CD, which acted as competitive inhibitors, caused the decline of the intrinsic fluorescence of the glycogen debranching enzyme (EC 3.2.1.33) [28]. Perhaps the main causes were the difference in size of enzyme molecular and steric hindrance at the site of enzyme. The fluorescence spectrum also exhibited blue shift (from 340 to 337 nm) of the maximum with an increase of β-CD. This reflected the fact that changes in the microenviroment around pullulanase resulted from the addition of β-CD and the native structure of pullulanase gradually unfolded. Some previous buried tryptophans were exposed to a less polar environment and the hydrophobicity around tryptophan residues increased. It also provided the evidence of formations between β-CD and pullulanase because of peculiar cyclodextrins hydrophobic cavum. Moreover, it was concluded that low concentration β-CD slightly dispersed molecular aggregation of pullulanase and enlarged the contact surface between substrate and pullulanase, which led to slight acceleration of enzymatic catalysis. But, higher concentration β-CD intensified the dispersion of pullulanase aggregation and furthermore changed the microenvironment around pullulanase, which gave rise to the loss of biological activity. From the above results, it was apparent that the addition of many β-CD caused a conformational change of pullulanase molecule.

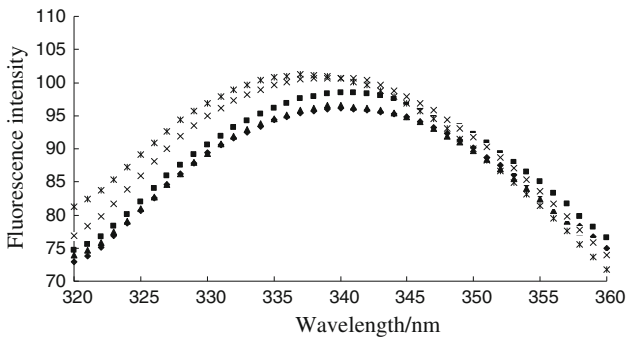

Fig. 2 Effects of β-CD on intrinsic fluorescence of pullulanase in the absence of β-CD (*filled diamond*) and in the presence of β-CD: 0.01 mM (*filled square*), 0.1 mM (*filled triangle*), 1 mM (*cross*) and 10 mM (*asterisk*). Each data point was the mean of three replicates

Effect of β-CD on secondary structure of pullulanase

As a rule, changes of catalytic activity for biological enzyme accompany by variations of the secondary structure and native configuration. The circular dichroism spectra can accurately interpret the changes of protein secondary structure in far-UV region [29, 30]. Changes of pullulanase secondary structure in the absence or presence of β-CD were detected by far-UV circular dichroism spectra and the secondary structure contents were calculated by means of K2D method. Table 1 showed the content changes of α-helix, β-sheet and random coil of pullulanase. For native pullulanase, the α-helix, β-sheet and random coil contents were approximately 15, 35 and 51%, respectively. The α-helix content in pullulanase decreased from 15 to 9% with the increase of β-CD from 0 to 10 mM. On the contrary, the β-sheet content increased from 35 to 43%. The random coil content slightly decreased from 51 to 48%. This implied that β-CD could effectively disrupt secondary structure of pullulanase in the presence of higher concentration. It is known that hydrogen bonding in α-helix

Table 1 The secondary structure of pullulanase induced by β-CD as determined from the far-UV circular dichroism spectra

[β-CD] (mM)	Secondary structure element (%)		
	α-Helix	β-Sheet	Random coil
0	15	35	51
0.01	15	35	51
0.1	14	37	50
1	13	38	50
10	9	43	48

is stronger than that in β-sheet. Hence, the conversion from α-helix to β-sheet conformation in the presence of β-CD was associated with the destabilization of pullulanase secondary structure. It was probably because β-CD entrapped aromatic amino acids residues of pullulanase into hydrophobic cavity so as to change the amino acid side chains and secondary structure elements. This was in accordance with the preceding results via analysis of intrinsic fluorescence. The binding in inclusion complexes appeared to derive from hydrogen bonding and van der Walls. Similar mode of binding has also been observed in the interaction between CDs and other enzymes including glycogen phosphorylase [4], pig pancreatic α-amylase [31] and soybean β-amylase [32]. However, Liu [28] reported that the secondary structure of debranching enzyme was not affected significantly by the binding of CDs. Therefore, it is clear in this instance that the interaction between enzymes and CDs occurs in different ways which depend mainly on the molecular structure and action pattern of enzymes.

Moreover, pullulanase is a member of α-amylase family, which are believed to possess a catalytic domain with the same basic three-dimensional fold, an $(\alpha/\beta)_8$-barrel. The respective contents of secondary structures and the ratio between α-helix and β-sheet are essential to maintain the conformation of enzymatic activity. As shown in Fig. 3, the ratio variation of α-helix to β-sheet was almost consistent with that of pullulanase RRA, especially in the presence of high concentration of β-CD. Both of them fell considerably with the increase of β-CD. The greater of the ratio variation of α-helix to β-sheet, the more likely its probability for pullulanase fold changed, which eventually led to the disruption of native conformation and the loss of enzymatic activity. Figure 3 further illustrated that the relationship between relative residual activity (RRA) and secondary structure. It indicated that the inactivation of pullulanase, especially in the presence of high concentration of β-CD, was a function of the degree of change in its secondary structure.

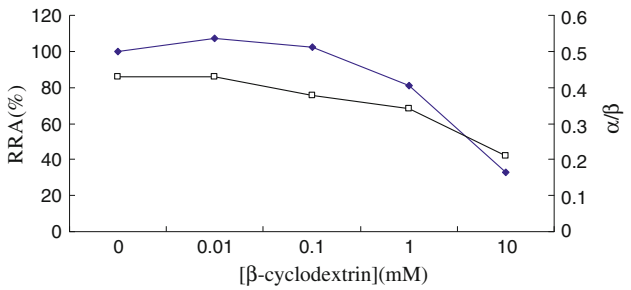

Fig. 3 The ratio variation (*open square*) of α-helix to β-sheet of pullulanase with various RRA Values (*filled diamond*) induced by β-CD

Conclusions

In this work, experimental studies were carried out to investigate the inhibition mechanisms of β-CD on pullulanase by exploring structure and microenvironment changes. Experimental data confirmed inhibition of pullulanase activity induced by β-CD in a concentration dependent manner. The fluorescence spectroscopy provided the evidence of inclusion formations between β-CD and pullulanase by the aid of the peculiar cyclodextrins hydrophobic cavum, which caused a conformational change of pullulanase molecule. The circular dichroism spectroscopy was further employed to verify that changes of pullulanase secondary structure depended on the β-CD concentration. The ratio of α-helix to β-sheet in pullulanase declined with the increase of β-CD, thus resulting in the loss of enzymatic activity. To sum up, the inhibition of pullulanase induced by β-CD was related to the change of structure and microenvironment. The present results were necessary and useful supplements for inhibition mechanism of β-CD on pullulanase.

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