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Study of conformational changes in glucoamylase *of Aspergillus awamori* nakazawa in presence of denaturants through CD-spectroscopy

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ABSTRACT

In present study, changes in the pattern or motifs of secondary structures of glucoamylase from *Aspergillus awamori* nakazawa in the presence of denaturants such as urea, guanidine-HCl (Gdn-HCl), and at different pH, were studied through CD-spectroscopy. It was observed that in native state glucoamylase entirely comprised of β-sheets. CD-spectra of glucoamylase in the presence of 6 M urea lost its spectral regular characteristic; and ellipticity became zero between 207 and 218 nm. With 0.5 M urea concentration glucoamylase showed approximately double negative ellipticity between 207 and 218 nm than the control, which indicates increase in β-sheets and decrease in random coil contents. But higher concentration of urea ($\geqslant$6 M) and Gdn-HCl ($\geqslant$3 M) completely unfold the enzyme. At lower concentration (0.1 M) of Gdn-HCl negative peak got shifted from 208 to 219 nm to a very sharp peak at 198 nm with lower intensity than the control. It was also observed that glucoamylase posses higher β-sheet in acidic media than in basic media. Glucoamylase remains active in a broad range of pH (3.0–11.0) and maximum activity was observed at pH 4.5. Activity of glucoamylase does not vary too much between pH 5.5 and 9.0. Conformational changes during wide range of pH were supported with its activity coincided.

1. Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3; GA) is an exohydrolase that catalyzes the release of β-D-glucose by hydrolyzing α-1,4- and α-1,6-glucosidic linkages at the non-reducing ends of raw or soluble starch and related oligosaccharides (Norouzian et al., 2006). Glucoamylase are used in industrial processes such as the production of glucose syrup and other food-processing applications. Although many fungal species are capable of producing glucoamylase under different growth conditions, industrial development of glucoamylase has focused on glucoamylases from *Aspergillus niger* (AnGA; identical to *Aspergillus awamori* GA) and *Rhizopus oryzae* (RoGA) because of their stability and high activity (Mertens and Skory, 2007).

In variety of industries enzymes are required in a form which can withstand high temperature, extreme pH and different denaturants. A better understanding of protein folding/unfolding processes allows us to predict more precisely a specific site or region critical to enzymes stability in presence of denaturants, high temperature and extreme pH of the media. Despite routine use of chemical denaturant, our understanding of the molecular mechanism by which chemical denaturants like urea or guanidinium cause a protein to unfold, is still rather limited. Circular dichroism (CD) is an excellent tool for rapid determination of the secondary structure and folding properties of proteins. Many researchers studied the changes in conformation of enzymes due to above factors through CD-spectroscopy in order to get highly stable enzymes.

Scheibe et al. (1990) had found no major difference in secondary structure or conformation between the two existing forms (oxidized and reduced) of chloroplast NADP-dependent malate dehydrogenase through circular dichroism and intrinsic protein fluorescence. The Gdn-HCl dependent unfolding of the enzyme was characterized by two transition midpoints: those of the reduced enzyme were lower by about 0.2 M Gdn-HCl compared to the oxidized enzyme. Denaturants increased the rate of reductive activation of NADP-malate dehydrogenase.

The effect of urea and Gdn-HCl on the activity of heart lactate dehydrogenase, glycerol-3-phosphate dehydrogenase, hexokinase, inorganic pyrophosphatase and glyceraldehyde-3-phosphate dehydrogenase were studied through CD-spectroscope in low-water systems by Garza-Ramos et al. (1992). Activities of these four enzymes were increased by 4–20 times by the denaturants.

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Xiao et al. (1993) studied the denaturation pattern of papain in the presence of Gdn-HCl. They observed that there was rapid decrease in activity with increasing concentrations of the denaturant followed by an intermediate stage of relatively little change from 1 to 2 M before complete inactivation at 4 M Gdn-HCl. At Gdn-HCl below 2 M enzyme activity was more sensitive to Gdn-HCl than noticeable conformation changes as seen by fluorescence and CD measurements.

Zhenyu et al. (2004) observed that a significant conformational transition to a secondary structure rich in β-sheet occurs when buffer concentration, salt concentration, pH or protein concentration were increased. An increase in salt concentration resulted in increase in β-sheet structure and self-association. Effect of denaturants such as urea, sodium dodecylsulphate (SDS) and Gdn-HCl on the structure and activity of 3-hydroxybenzoate-6-hydroxylase was studied by Sumathi and Dasgupta (2006) using intrinsic fluorescence and far and near-UV-CD-spectroscopy. They reported that far-UV-CD spectrum of the enzyme didn't show any appreciable alterations in presence of urea, SDS and Gdn-HCl, suggesting no major conformational changes in the α-helical secondary structure of the enzyme. However, treatment with 2 M urea as well as 0.01% SDS resulted in almost complete loss of activity and reduction in the intensity of near-UV-CD spectrum, especially at 280 nm. Treatment with 20 mM Gdn-HCl also resulted in about 70% loss in activity.

In present work, study of the conformational changes and behaviour of glucoamylase, produced under SSF by locally isolated strain of *A. awamori* nakazawa (MTCC 6652), in presence of different denaturants such as urea, guanidine-HCl (Gdn-HCl) and pH has been carried out through CD-spectroscopy.

2. Methods

2.1. Chemicals

The chemicals used for the experiments were all of analytical grade.

2.2. Microorganism

A locally isolated *A. awamori* nakazawa (MTCC 6652) was used for the present investigation. This strain was maintained on 2% malt-extract agar slants at 4 °C.

2.3. Glucoamylase production and purification

Glucoamylase was produced through solid state fermentation by locally isolated *A. awamori* nakazava (Negi and Banerjee, 2006) and purified enzyme was recovered by ion exchange and size exclusion chromatography (Negi and Banerjee, 2009). Purified glucoamylase was used for present study.

2.4. Glucoamylase assay

Glucoamylase activity was measured by Bernfeld method (1955). Absorbance had been measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that released one micromole of reducing sugar as glucose in one minute under the assay conditions.

2.5. Effect of pH on glucoamylase activity

The rate of digestion of starch with purified corresponding enzymes were evaluated at various pH values. In order to acquire different pH conditions 0.5 ml of 1% starch solution was taken as substrate and reaction volume was adjusted with 0.5 ml buffer of different pH, and activities were calculated at different pH.

2.6. Effect of denaturant on glucoamylase activity

The influence of denaturant like urea and Gdn-HCl on amylase were studied in the range of 0.25–6.0 M and 5 mM–3 M, respectively, by incubating enzyme for 30 min. The results were compared with that of the control (without any urea) and expressed as percent relative activity.

2.7. Circular dichroism spectroscopy

Secondary structure of native enzyme and enzyme treated with urea and Gdn-HCl of different concentration (5 mM–6 M) were analyzed through circular dichroism spectrophotometer (Jasco-810) in the UV range of 190–290 nm at room temperature with a scanning speed of 50 nm/min in the cell of 0.1 cm length. Purified amylase with 0.88 mg/ml of protein concentration was used for analysis. While taking reading acetate buffer (0.1 M) in which enzymes was dissolved, was taken as base line to subtract the spectra of buffer. Secondary structure fraction for glucoamylase were determination from far-UV-spectra using the algorithm from the reference: Yang.jwr.

3. Results and discussion

3.1. Secondary structure determination of glucoamylase

CD-spectra in the far-UV region (190–230 nm) provide important and quantitative information about protein secondary structure. Homogeneous and purified sample of glucoamylase produced through SSF by *A. awamori* nakazawa were subjected for CD-spectroscopic analysis to understand the mechanism by which chemical denaturants like urea, guanidine and pH of the media cause a protein to unfold or denature.

3.2. Effect of urea on glucoamylase activity

Urea plays an important role in unfolding and refolding of the protein. Therefore, influence of urea on glucoamylase activity was studied in the range of 0.25–6 M by incubating enzymes for 30 min. Although, urea is known to cause denaturation of enzyme but in the present study it caused activation of purified glucoamylase at a concentration of 0.5 M, as shown in Fig. 1. At higher concentration of urea, enzyme activity decreased slowly and complete inhibition took place at 6 M urea concentration. The probable reason for this could be that at lower concentration urea unfolds the tertiary structure to the extent that hidden active sites present in-

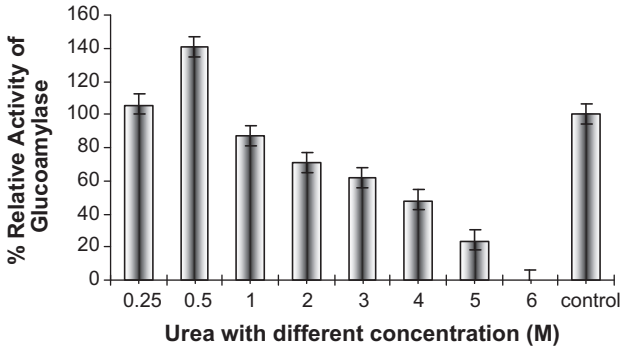

Fig. 1. Effect of urea concentration on amylase activity.