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Effect of glycation on stability and kinetic parameters of thermostable glucoamylase from *Aspergillus niger*

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Abstract

The effect of glycation on properties of thermostable glucoamylase was studied by incubating purified glucoamylase with maltodextrin at 60 °C. Glycation occurred during the incubation and was assessed by 5-(hydroxymethyl)-2-furfuraldehyde (HMF) released from acid hydrolysis of the glycated protein. Stability and kinetic parameters of the glycated glucoamylase and the intact enzyme were compared. The glycated enzyme was more resistant to the heat but glycation did not strongly affect pH stability and p*I* value significantly. In the presence of maltose as substrate, the K_m value of the glucoamylase glycated by maltodextrin was lower than that of the intact enzyme. This indicated a greater affinity of the glycated enzyme for maltose. In the presence of maltodextrin as substrate, glycation led to increases in the rate of hydrolytic reaction. Moreover, glycation resulted in a higher efficiency of glucoamylase to convert the substrate into glucose. This might be due to a greater conformational flexibility of the glycated glucoamylase.

Keywords: Glycation; 5-(Hydroxymethyl)-2-furfuraldehyde (HMF); Glucoamylase; Thermostable; *Aspergillus niger*; Kinetic parameters

1. Introduction

Glucoamylase (EC 3.2.1.3; 1, 4-α, D-glucan glucohydrolase) is an exoglucosidase that catalyzes the release of glucose units from a non-reducing end of maltodextrin chains. In general, glucoamylase is a multidomain enzyme consisting of a catalytic domain connected to a starch binding domain by an *O*-glycosylated linker region [1–4]. Glucoamylases from various species of *Aspergillus* widely used in the manufacture of glucose syrup are thermostable with optimum temperature of 58–65 °C, optimum pH of 4.0–4.5 and pH stability in a range of 3.5–5.5 [2,5,6]. In glucose syrup production, starch is first liquefied by α-amylase and further saccharified by the glucoamylase to yield glucose. The saccharification process is carried out at 55–60 °C for 48–92 h, yielding glucose syrup with dextrose equivalence (DE) of 97–98 [5–7].

Under conditions of high reducing sugar concentration and high temperature, proteins in general can readily undergo glycation [8–10]. This non-enzymic modification occurs when a free amino group on amino acid residues such as N-terminal amino acid, Lys, His, and Arg residues of the protein undergo condensation with a carbonyl group of a reducing sugar. The glycated protein in the form of Amadori's products then are further dehydrated, leading to structural alteration and intramolecular cross-linking of the protein molecule [9–11]. Numerous in vivo and in vitro investigations have demonstrated the occurrence of glycation in various enzymes [12–14]. Since industrial glucoamylase used in saccharification processes is employed under high reducing sugar and high temperature conditions, glycation might be involved in inactivation of the glucoamylase. In this work, the alteration of kinetic parameters of industrial glucoamylase due to glycation have been investigated, and have explained the effect of the glycation on enzyme functions and its kinetic parameters.

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2. Materials and methods

2.1. Materials

Glucoamylase (DAIZYME; Daiwa Kasei K.K., Japan), produced from *Aspergillus niger*, was a gift from Cinnamon Company Ltd. (Thailand). Food grade maltodextrin (MORREX, DE = 15–19) was purchased from Stamford Food Industries (Malaysia). D-Glucose was purchased from BDH (England). The 5-(Hydroxymethyl)-2-furfuraldehyde (HMF) standard was obtained from Fluka (Buchs, Switzerland). Bovine serum albumin (Fraction V) was purchased from Sigma (MO, USA). All chemicals were of the best grade available supplied from Merck (Darmstadt, Germany) unless otherwise stated.

2.2. Purification of glucoamylase

Purified glucoamylase was prepared by a modified method of Odibo and Ulbrich-Hofmann [15]. One milliliter of the crude enzyme containing 74.91 mg of protein was applied to a Q-Sepharose Fast Flow column (1.6 cm × 40 cm, Amersham Pharmacia Biotech Ltd., UK) previously equilibrated with 20 mM acetate buffer (pH 4.5). Elution was performed with a 400 ml linear gradient (0.3–0.5 M) of NaCl at a flow rate of 1 ml/min. Three milliliters of each fraction was collected using a fraction collector (Model 2110; Bio-Rad, CA, USA). After that, the enzyme activity was determined. The active fractions were pooled and concentrated by ultrafiltration (VEVACELL 250; Vivascience, Germany). The partially purified enzyme was further purified with a Phenyl-Sepharose CL-4B column (2 cm × 40 cm, Sigma). Before loading the enzyme onto the column, ammonium sulphate was added to the final concentration of 1.0 M. After equilibration of the column with 20 mM acetate buffer (pH 4.5, containing 1.0 M ammonium sulphate), the enzyme concentrate was applied to the column. The protein was then eluted with a 250 ml linear gradient from 1 to 0 M ammonium sulphate in the same buffer. Three milliliters of each fraction was collected and the enzyme activity was determined. The active fractions were pooled and concentrated by ultrafiltration. The purity of the enzyme was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [16], and shown to be homogeneously pure when the enzyme was applied to a Phenyl-Sepharose CL-4B column. SDS-PAGE indicated a molecular weight of 77.6 kDa. This was consistent with previous reports [17,18]. The purified glucoamylase (PG) was used to study the hydrolysis of maltodextrin.

2.3. Hydrolysis of maltodextrin by glucoamylase

Reaction volumes (15 ml) were prepared to contain glucoamylase (0.1 mg protein/ml) and maltodextrin (350 mg/ml) in 20 mM acetate buffer (pH 4.5) under aseptic conditions. The reaction mixtures were incubated in a shaking water bath at 150 rpm, 60 °C. Samples were withdrawn at specific time intervals (i.e. 0, 12, 24, 48, and 72 h) and the enzyme activity was terminated by rapid cooling to 4 °C. The sample aliquot (250 μl) was added with 4% perchloric acid (250 μl) and kept at −20 °C for determination of the reducing power and glucose content. The remaining samples were centrifuged (4500 × *g*, 45 min, 4 °C) to exclude aggregate prior to protein assay. Portions were dialyzed against 20 mM acetate buffer (pH 4.5, 4 °C) to exclude the maltodextrin and sugar product and then concentrated by ultrafiltration (Ultrafree-15; Millipore, USA), prior to the measurement of the degree of glycation.

2.4. Preparation of glycated enzyme

Glycated glucoamylase was prepared by a modified method of Arai et al. [12]. One hundred milliliters of glycated glucoamylase was prepared by incubating the purified enzyme (0.1 mg protein/ml, 66 units/mg protein) with 350 mg/ml maltodextrin solution (20 mM acetate buffer, pH 4.5) at 60 °C, 24 h. The reaction mixture was then centrifuged at 4 °C, 4500 × *g* for 45 min to remove protein precipitate. After the supernatant was dialyzed against 20 mM acetate buffer (pH 4.5), the solution was concentrated by ultrafiltration (Ultrafree-15; Millipore, USA). This glycated enzyme (gPG) was used to study enzyme stability.

2.4.1. Heat stability of glycated enzyme

One milliliter of gPG (0.1 mg protein/ml) was incubated in 20 mM acetate buffer (pH 4.5) at 60 °C. Samples were taken at specific time intervals (i.e. 0, 2, 4, and 6 h) and assayed for total protein and enzyme activity. PG was used as a control experiment. The glycated glucoamylase by glucose was prepared as described above to compare the heat stability.

2.4.2. pH stability of glycated enzyme

One milliliter of the enzyme (gPG and PG, 0.1 mg protein/ml) was incubated in 20 mM acetate buffer (pH 3.5, 4.5, 5.5) and 20 mM phosphate buffer (pH 6.5) at 60 °C for 1 h. Total protein and enzyme activity were determined.

2.5. Effect of glycation on enzyme kinetic parameters

Kinetic parameters were determined at optimum condition (pH 4.5, 60 °C). The reaction was conducted by mixing a fixed activity of enzyme (0.73 U/ml substrate solution) with different substrate concentrations (maltose or maltodextrin). The mixture was withdrawn at specific time intervals to analyze glucose concentration by HPLC. The kinetic parameters were determined by fitting the initial rate data to the Michaelis–Menten equation by non-linear regression using SigmaPlot software (SPSS Inc., USA). The values of K_m and maximal velocity (V_{max}) were calculated based on Lineweaver–Burk plot.