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Characterization and solvent engineering of wheat β-amylase for enhancing its activity and stability

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ABSTRACT

The kinetic and thermodynamic parameters of wheat β-amylase (WBA) were characterized and various additives were evaluated for enhancing its activity and thermostability. WBA activity was examined by neocuproine method using soluble starch as substrate. The Michaelis constant (K_m) and molecular activity (k_{cat}) were determined to be 1.0 ± 0.1% (w/v) and 94 ± 3 s^{-1}, respectively, at pH 5.4 and at 25 °C. The optimum reaction temperature (T_{opt}) for WBA activity was 55 °C and the temperature (T_{50}) at which it loses half of the activity after 30-min incubation was 50 ± 1 °C. Modifications of the solvent with 182 mM glycine and 0.18% (w/v) gelatin have increased the T_{50} by 5 °C. Glycerol, ethylene glycol, dimethylformamide (DMF) and dimethyl sulfoxide have also slightly enhanced the thermostability plausibly through weakening the water structure and decreasing the water shell around the WBA protein. Ethanol and DMF activated WBA by up to 24% at 25 °C probably by inducing favorable conformation for the active site or changing the substrate structure by weakening the hydrogen bonding. Its half-life in the inactivation at 55 °C was improved from 23 to 48 min by 182 mM glycine. The thermodynamic parameters indicate that WBA is thermo-labile and sufficient stabilization was achieved through solvent modification with additives and that the heat inactivation of WBA is entropic-driven. It is suggested that WBA could be applied more widely in starch-saccharification industries with employing suitable additives.

1. Introduction

β-Amylase hydrolyses the α-1,4-glucan bonds in amylosaccharide chains from the non-reducing ends and generates maltose. It has considerable application in the production of high maltose syrups together with starch debranching enzymes and α-amylases. β-Amylase has been well characterized in higher plants [1–3] and micro-organisms [4–7]. However, the well-characterized β-amylases are neither active nor stable at higher temperatures >65 °C [8]. In many findings, α-amylase is more stable compared to β-amylase of the same origin [9–11]. Enzymes may easily be denatured by slight change of the environmental conditions such as temperature, pressure, pH, and ionic strength [9]. Nevertheless, stabilization of the enzymes could be achieved in several ways: screening for more stable ones (favorably from thermophiles and extremophiles), chemical modification, site-directed mutagenesis, immobilization and solvent engineering or modifying the enzyme reaction conditions with stabilizing additives [12–17]. In solvent engineering, selection of appropriate additives is dependent on the nature of the enzyme and there are no established rules to select effective additives for improving enzyme functions [18]. The thermostability of β-amylase has been substantially enhanced by modifying the solvent with additives [18,19].

Different amylases give oligosaccharides with specific lengths of end products. For this reason, amylases with unique properties need to be studied for various applications in starch-saccharification for production of food and bio-ethanol [20]. Unlike soybean, barely and sweet potato, wheat was not the common source of β-amylase for starch-saccharification so far. However, the sources of β-amylase supply have drastically changed due to the escalating prices of the major sources like soybean. Wheat β-amylase (WBA) is prepared from wheat bran, which is an industrial by-product in the production of wheat starch and gluten. It is a cheaper alternative source of β-amylase for industries. Nevertheless, it is lower in thermostability as compared with β-amylases of other crops and microbes. For instance, the optimum temperature (T_{opt}) of Clostridium thermosuiphurogenes β-amylase is 75 °C [8]; the temperature at which it loses half of its activity after 30-min incubation (T_{50}) of barely β-amylase is 56.8 °C and that of soybean β-amylase is 63 °C [21] while the T_{opt} and T_{50} after 30-min incubation of WBA are 55 and 50 °C, respectively (data obtained in this study).

Therefore, enhancing the activity and thermostability of WBA has an excellent prospect for starch-saccharification industries. In this study, we used a commercially-available WBA preparation, Himaltosin, without further purification because it is already

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purified from other protein contaminants and utilized industrially. In this paper, we describe the kinetic and thermodynamic properties of WBA and improvement in its activity and thermostability via solvent engineering using various additives. This suggests that WBA would be likely applicable to a wide range of starch-saccharification industries.

2. Materials and methods

2.1. Materials

Himaltosin GS (Lot 2S24A), a commercial preparation of WBA, was purchased from HBI Enzymes (Osaka, Japan). This preparation was filtered with a Millipore membrane filter (Type HA; pore size: 0.45 μm) and used without further purification. According to the manufacturer, the Himaltosin preparation contains 90% starch as a stabilizer, and almost all of the protein is β-amylase and α-amylase was not detected. Himaltosin was suspended to 20 mM sodium acetate buffer (pH 5.4) at 25 °C to be 0.3 mg/ml. In this paper, this buffer was hereinafter referred to as buffer A. The WBA protein content was expected to be 0.03 mg/ml in the suspension and it was followed by filtration with the Millipore membrane filter. However, the protein concentration in the filtrate was less than 10% of the expected content, suggesting that >90% of the WBA protein was remained with starch on the filter. The WBA concentration was determined spectrophotometrically in buffer A using the absorptivity value (A) of 1.40 ± 0.02 at 281 nm with a 1.0-cm light-path for the WBA solution at the concentration of 1.0 mg/ml [22]. The molecular mass of 57.5 kDa for WBA was used to determine the molar concentration of WBA (see Sections 2.3 and 3.1). Under the standard condition in this study, the concentration of WBA in the enzyme-reaction solution was set to 15.0–30.0 nM. The starch concentration due to the stabilizer starch (0.027%, w/v) was completely removed by filtration with the Millipore membrane filter. Soluble starch (Lot M7H1482) as substrate and maltose (Lot M1F7568) as standard for the activity assay were obtained from Nacalai Tesque (Kyoto, Japan). The substrate has a weight-average molecular weight of 1.0×10^6 according to the manufacturer, and thus the average degree of polymerization of the glucose unit is estimated to be 6000. Neocuproine–HCl (2,9-dimethyl-1, 10-phenanthroline, Lot 032K2533) as coloring reagent B in the neocuproine method was from Sigma (St. Louis, MO, USA). Coloring reagent A (0.38 M Na_2CO_3, 1.8 mM $CuSO_4$, and 0.2 M glycine) in the neocuproine method and all other chemicals were purchased from Nacalai Tesque. All enzyme reactions were carried out in buffer A, pH 5.4.

2.2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a 10% polyacrylamide gel under reducing conditions [23]. The Himaltosin preparation was suspended in buffer A to the concentrations of 1, 2, and 5% (w/v). The solution was filtered with a Millipore membrane filter (Type HA) and reduced by treatment with 10 mM dithiothreitol (DTT). The solution was applied to SDS-PAGE with a constant voltage of 150 V for 80 min. Proteins were stained with Coomassie Brilliant Blue R-250. The molecular-mass marker kit consisting of rabbit muscle phosphorylase *b* (97.2 kDa), bovine serum albumin (66.4 kDa), hen egg albumin (44.3 kDa), and hen egg white lysozyme (14.4 kDa) was a product of Takara Bio (Otsu, Japan).

2.3. Measurement of enzyme activity

Various initial concentrations of the soluble starch substrate [0.00, 0.09, 0.45, 0.90, 1.13, 1.35, 1.80, 2.02, 2.25, 2.70, 2.93, and 3.15% (w/v)] in the reaction solution were prepared in buffer A at 25 °C. The WBA solution in the same buffer was filtered with a Millipore membrane filter (Type HA) and kept in ice water for immediate use. The various concentrations of starch were hydrolyzed by WBA (30.0 nM) for 0, 2.5, 5.0, 7.5, and 10.0 min at 25 °C. The reaction was stopped by adding 300 μl of 0.1 M NaOH into 100 μl of the enzyme-reaction solution. The amount of the reducing sugar in the enzyme-reaction solution was determined by the neocuproine method as follows [24]. Reagent A and reagent B, 250 μl each, were mixed with 50 μl of the enzyme-reaction solution, boiled for 8 min, and diluted with 550 μl of water after cooling in ice water. The activity was measured at 450 nm using a Beckman-Coulter DU 800 spectrophotometer (Batavia, IL, USA) [25,26]. The enzyme activity was determined by measuring the velocity (v) of reducing sugar production, and the reaction velocity was analyzed by the Michaelis–Menten kinetics. The maximum velocity (V_{max}) and Michaelis constant (K_m) were obtained from the v vs. the substrate concentration ([S]) plots using KaleidaGraph 4.1 (Synergy Software, Reading, PA, USA). The molecular weight of WBA is 57,500 [27] and was used for the evaluation of the molecular activity (k_{cat}) (see Section 3.1).

2.4. The optimum temperature

Starch solution (900 μl) in buffer A was mixed with the WBA solution (0.150 μM; 100 μl) in the same buffer for 0, 2.5, 5.0, 7.5, and 10.0 min at 25 °C after incubation at 15, 25, 35, 45, 55, 65, and 75 °C in a water bath for 10 min. The initial concentrations of starch and WBA in the reaction solution were 1.80% and 15.0 nM, respectively. The reaction was stopped by adding 300 μl of 0.1 M NaOH into the enzyme-reaction solution (100 μl). Then the enzyme activity was determined by the neocuproine method (see above). The optimal reaction temperature at which WBA exhibited the highest activity was referred to as the optimal temperature (T_{opt}).

2.5. Thermal inactivation of WBA

The substrate and WBA solutions were prepared in buffer A. The enzyme solution was incubated at 25, 35, 45, 55, and 65 °C for 10, 20, and 30 min and cooled at 25 °C for 3 min in a water bath. The substrate solution (2.00%, w/v; 900 μl) was mixed with the heat-treated WBA solution (0.300 μM; 100 μl) at 25 °C and incubated for 0, 2.5, 5.0, 7.5, and 10.0 min. The initial concentrations of substrate and enzyme in the reaction solution were 1.80% and 30.0 nM, respectively. The activity was assayed as aforementioned by the neocuproine method and the first-order rate constant k of the thermal inactivation was determined assuming pseudo-first order kinetics by plotting ln (v/v_o) against the heat-treatment time (t) (Eq. (1)), where v is the initial reaction velocity of the enzyme with heat treatment at each incubation temperature and v_o is that obtained without heat treatment and at 25 °C. The activation energy E_a of the thermal inactivation was obtained by the Arrhenius plot (Eq. (2)), and the standard Gibbs energy difference of activation for thermal inactivation ($\Delta G^{\circ\ddagger}$), the standard enthalpy difference of activation ($\Delta H^{\circ\ddagger}$), and the standard entropy difference of activation ($\Delta S^{\circ\ddagger}$) were obtained from the Eyring plot according to Eqs. (3) and (4) [24,28].

$$\ln \frac{v}{v_o} = kt \tag{1}$$

$$\ln k = -\frac{E_a}{R}\frac{1}{T} \tag{2}$$

$$\Delta G^{\circ\ddagger} = -RT\left[\ln\left(hk/k_B T\right)\right] \tag{3}$$

$$\ln \frac{hk}{k_B T} = -\frac{\Delta H^{\circ\ddagger}}{RT} + \frac{\Delta S^{\circ\ddagger}}{R} \tag{4}$$

where k_B, h, and R are the Boltzmann, Plank, and gas constants, respectively. T is temperature in Kelvin.

2.6. Activation and thermostablization of WBA using additives

The WBA solution in buffer A was mixed and incubated with equal volume of various additives in the same buffer at 25, 45, 55, and 65 °C in a water bath for 30 min before hydrolyzing soluble starch. The initial concentrations of WBA and substrate in the reaction solution were 30.0 nM and 1.80%, respectively. The additive concentrations in the reaction solution were: 45.5, 91, 182, and 364 mM glucose, NaCl, and glycine; 45.5 and 91 mM L-arginine; 0.9, 1.8, 3.6, and 45.5 mM of L-aspartate; 45.5 and 91 mM of L-cysteine and glutathione (GSH); 0.18 and 0.45% (w/w) gelatin; 0.91, 1.82, and 5.5% (w/w) ethanol and 2-methyl-2-butanol (2M2B); 0.45 and 2.7% (w/w) dimethyl sulfoxide (DMSO) and dimethylformamide (DMF); and 0.91 and 5.5% glycerol, ethylene glycol (EG), and β-mercaptoethanol (βME). Their effects on activation and thermal stabilization of WBA were examined. The enzyme–additive mixture solution (0.20 ml) was diluted with water (0.55 ml), and the enzyme activity was measured by the neocuproine method.

3. Results

3.1. Kinetic parameters of WBA

The WBA preparation, Himaltosin, shows a single band in SDS-PAGE with molecular mass of 57.5 kDa (Fig. 1). This value is in good agreement with those (54.0–64.2 kDa) so far reported [22,27]. In this paper, we used 57.5 kDa for the molecular mass of WBA. This indicates that the Himaltosin preparation is composed of solely β-amylase as a protein component, although it contains 90% (w/w) starch as a stabilizer. Substantially, there was no starch carried over from the stabilizer starch into the reaction solution as examined by the starch–iodine reaction. Therefore, the enzyme preparation was treated as WBA without further purification.

The initial velocity (v) of the WBA-catalyzed hydrolysis of different concentrations of soluble starch was examined in buffer A at 25 °C at the enzyme concentration of 30.0 nM. The dependence of v on substrate concentration exhibited the Michaelis–Menten profile (Fig. 2). The K_m, V_{max}, and k_{cat} were determined to be 1.0 ± 0.1% (w/v); 2.8 ± 0.1 μM s^{-1}, and 94 ± 3 s^{-1}, respectively, by fitting the experimental data to the Michaelis–Menten equation.