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Physicochemical and kinetic properties of a high salt tolerant *Aspergillus flavus* glucoamylase

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ABSTRACT

Amylases with excellent biochemical properties, broad starch degradation ability and high glucose yield are ultimate industrial requirements for starch processing. Here, extracellular glucoamylase produced from *Aspergillus flavus* under submerged fermentation was purified to apparent homogeneity by combination of 0–60% ammonium sulphate precipitation, ion-exchange chromatography and gel filtration. The purified enzyme has a monomeric molecular weight of 63 kDa and exhibits optimum pH and temperature at 5.5 and 50 °C, respectively. The enzyme was acidic thermostable at pH 4.0–7.0 retaining about 62% of initial activity after 1 h of incubation at 60 °C. Using raw cassava starch as substrate, the enzyme has a K_m value of 0.72 mg/mL with V_{max} of 12.48 μmol/min/mg. This glucoamylase exhibited high degree of salt tolerance with residual activity above 65% after 24 h incubation in 2.0 M NaCl solution. The activity of the purified enzyme was enhanced by Ca^{2+}, Mg^{2+}, Na^{+} but inhibited by Al^{3+}, Cu^{2+}, Hg^{2+}, Ethylenediamine tetraacetic acid (EDTA) and Urea. The results from this study affirm that this fungal glucoamylase possess good biochemical characteristics for raw-starch digestibility suitable for biotechnological application.

1. Introduction

Industrial revolution in the starch-utilizing industries has necessitated the use of efficient biocatalysts in starch liquefaction and saccharification as against the crude acid hydrolysis method. These starch-degrading enzymes known as amylases, account for about 25% of the enzymes used in the industry (Kumar and Satyanarayana, 2009; Nwagu and Okolo, 2011) and are of great importance due to their wide application in food, pharmaceutical, paper, chemical and textile industries (Selvakumar et al., 1996; Ominyi, 2013).

Glucoamylase (1,4-α-D-glucan glucohydrolase; EC 3.2.1.3), one of the three best known amylases has particularly received industrial usefulness in starch degradation to yield soluble sugars widely used by many food industries for the production of glucose and fructose syrups (Van Der Veen et al., 2005; Riaz et al., 2007) and in the production of bioethanol (Pervez et al., 2014). It is said to be the most important industrial enzyme because of its widespread uses together with α-amylases and debranching enzymes in the saccharification of starch (Kumar and Satyanarayana, 2009).

Glucoamylase hydrolyzes 1,4-linked α-D-glucosyl residues successively from the non-reducing end of oligosaccharide and polysaccharide chains with the release of β-D-Glucose (Jafari-Aghdam et al., 2005). This exo-acting carbohydrase can also hydrolyze α-1,6 bonds on amylopectin branching points of starch molecules and at slower pace, almost all α-glycosidic bonds including α, β-(1,1)-, α-(1,2)-, and the rare α-(1,3)- linkages, except α,α-trehalose (Kumar and Satyanarayana, 2009; Slivinski et al., 2011), thus, promoting a complete starch hydrolysis into glucose if incubated for extended period of time (Karim et al., 2016).

Many species of animals, plants and microorganisms are capable of producing starch degrading enzymes; however, filamentous fungi constitute the major industrial source among all microorganisms (Riaz et al., 2007). The use of microorganisms as potential sources of industrially viable enzymes has gained popularity in the field of biotechnology owing to the fact that these microorganisms are ubiquitous in nature and they are known to reproduce fast when grown on the required media concomitantly secreting the enzyme of interest (Saha and Zeikus, 1989). They are easy to manipulate to obtain enzymes of desired characteristics (Renge et al., 2012; Shruti et al., 2013) and in economical bulk production capacity (Acharya et al., 2014).

Though, amylases have been characterized from different species, only a selected few are able to hydrolyze raw starch (Van Zyl et al., 2012). Raw starch degrading enzymes that can both liquefy and saccharify raw starch will significantly reduce the energy requirements and simplify the production of starch-based biofuels (Robertson et al., 2006). As such, there is a continuous search for new sources of

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glucoamylases with excellent characteristics that can be used alone or in combination with α-amylase and also their compatibility with industrial strains of yeast for efficient hydrolysis and fermentation of starch-based biomass to liquid biofuel (Aydemir et al., 2014). This paper describes the biochemical characterization of a thermostable, high salt-tolerant and raw-starch degrading glucoamylase purified from an *Aspergillus* species obtained from the soil of a cassava processing industry in Akure, Southwest Nigeria.

2. Materials and methods

2.1. Materials

Unprocessed raw cassava starch and corn starch were obtained from Oja-oba, a local market in Akure, Southwest Nigeria and authenticated at the Department of Crop, Soil and Pest Management, The Federal University of Technology, Akure, Nigeria (FUTA). Soluble starch (Cat #S9765), Dextran (Cat #31398), Amylose (Cat #A0512), Potato soluble starch (Cat #S2004), Sephadex G-100 (Cat #G100120), Carboxyl methyl (CM)-Cellulose (Cat #C0806), Acetic acid (Cat #320099), Sodium acetate (Cat #2889), Potassium phosphate monobasic (Cat #5655), Di-potassium hydrogen phosphate (Cat #3786) Trizma base (Cat #T1503), Urea (Cat #U5378), Glycine (Cat #G8898) were acquired from Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA. All other reagents and chemicals used were of analytical grade purchased from local accredited suppliers.

2.2. Microorganism

Microorganism previously isolated from the soil environment of a cassava processing industry in Akure, Southwest Nigeria (coordinates 7°10′N 5°05′E) was obtained from the culture collection of Enzyme and Microbial Technology Laboratory, Department of Biochemistry, The Federal University of Technology, Akure, Nigeria. The fungal species was maintained on Potato dextrose agar (PDA) slants, stored at 4 °C and sub-cultured periodically on new agar slants throughout the period of study.

2.3. Plate screening of microorganism for amylolytic activity

The organism was inoculated onto sterilized starch agar medium prepared by adding 1% (w/v) soluble starch to growth medium. Plates were incubated at 37 °C for 72 h, after which they were stained with a solution containing potassium iodide, KI (0.1% w/v)/ I_2 (0.1% w/v) in 1.0 M HCl for 15 min and later de-stained with distilled water. A clear halo zone around the colonies indicated amylolytic activity.

2.4. Production of glucoamylase in submerged fermentation

Glucoamylase was produced at optimized conditions of submerged fermentation in Erlenmeyer flasks containing formulated liquid mineral media consisting of yeast extract 0.67%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4$ 0.01%, $CaCl_2 \cdot 2H_2O$ 0.01%, KH_2PO_4 0.02%, $(NH_4)_2SO_4$ 0.333% and soluble starch 1% with pH adjustment to 5.0. A set of shake-flasks containing five Erlenmeyer flasks (250 mL) was used for the enzyme production. Flasks were plugged with cotton wool and sterilized by autoclaving for 15 min at 121 °C and 1.1 kgf/cm^2. After sterilization, the flasks were cooled and inoculated with inoculum having 10^6–10^7 spores/mL at a concentration of 10% (v/v) from freshly prepared seed culture from the slant. The flasks were incubated at 30 ± 1 °C for 72 h under a constant shaking condition at 150 rpm. At the end of fermentation, the crude enzyme was extracted from growth media by filtration through a Whatman no 1 paper. The filtrate was centrifuged at 10,000×*g* for 20 min at 4 °C to remove the suspended particles. The supernatant is herewith referred to as the crude enzyme.

2.5. Enzyme activity assay and determination of protein content

Glucoamylase activity was determined by a standard assay procedure earlier described by Cereia et al. (2000). Briefly, enzyme solution (100 µl) was added to a test tube containing 100 µl of 1% (w/v) soluble starch buffered with 0.05 M acetate buffer, pH 5.5 and incubated at 60 °C in a water bath for 10 min. The amount of glucose released was measured using the dinitrosalicylic acid (DNSA) method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme required to liberate one micromole of reducing sugar per minute under assay conditions.

The protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard protein.

2.6. Purification of enzyme

The crude enzyme from *Aspergillus flavus* was brought to 60% saturation, centrifuged and the pellet was dialyzed extensively against 50 mM sodium acetate buffer (pH 5.5) at 4 °C. The dialysate was applied to a CM-Cellulose column (2.5×40 cm, flow rate of 60 mL/h) previously equilibrated and washed with the same buffer, the bound protein fractions were eluted with linear gradient of sodium chloride (0–1 M). Protein concentration was monitored at 280 nm and glucoamylase activity was tracked in the eluted fractions. The active fractions were pooled, and concentrated by 10 kDa concentrator before loading onto a Sephadex G-100 gel filtration column (2.5×75 cm) previously equilibrated and washed with 50 mM acetate buffer (pH 5.5) and adjusted to a final flow rate of 15 mL/h.

2.7. Determination of molecular mass

SDS-PAGE was performed on a 12% polyacrylamide gel using Tris-Glycine-SDS buffer system according to the method of Laemmli (1970) to determine the purity/homogeneity and the sub-unit molecular mass of the purified glucoamylase. Electrophoresis was performed at 80 V for 6 h using the Bio-Rad electrophoresis system (Bio-Rad, UK). The gel was stained with Coomassie brilliant blue and the protein bands were observed after destaining the gel.

2.8. Physicochemical characterization

2.8.1. Effect of pH on enzyme activity and stability

The optimum pH for activity of the purified glucoamylase was determined by performing the enzyme activity assay earlier described at different buffer solution pH using the following buffers (50 mM): Glycine-HCl buffer (pH 2.0–3.0), sodium acetate buffer (pH 4.0–5.5), potassium phosphate buffer (pH 6.0–7.5) and Tris-HCl buffer (pH 8.0–9.0). The effect of pH on enzyme stability was determined by pre-incubating the purified enzyme in various relevant buffer solutions, pH 4.0–7.0, at room temperature for 2 h with periodic sampling every 20 min. The residual activity of the enzyme was thereafter measured under standard assay conditions earlier described.

2.8.2. Effect of temperature on enzyme activity and stability

The effect of temperature on glucoamylase activity was determined at different temperatures (30–100 °C) in sodium acetate buffer solution, pH 5.5 under standard assay conditions previously described. Thermal stability of the purified glucoamylase was determined by incubating it in 50 mM sodium acetate buffer (pH 5.5) at various temperatures (30–90 °C) for 2 h with periodic withdrawal of aliquot of enzyme every 20 min. Residual activity was measured under standard assay conditions.

2.8.3. Effect of metallic ions and EDTA on enzyme activity

To determine the effect of metal ions (K^+, Mg^{2+}, Hg^{2+}, Cu^{2+}, Ca^{2+},