Purification and Biochemical Characterization of a Detergent Stable α-amylase from *Pseudomonas stutzeri* AS22

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Received: 27 December 2012 / Revised: 1 May 2013 / Accepted: 5 May 2013 © The Korean Society for Biotechnology and Bioengineering and Springer 2013

Abstract This study reports the purification and biochemical characterization of a novel maltotetraose-forming-α-amylase from *Pseudomonas stutzeri* AS22, designated PSA. The *P. stutzeri* α-amylase (PSA) was purified from the culture supernatant to homogeneity by Sepharose mono Q anion exchange chromatography, ultrafiltration and Sephadex G-100 gel filtration, with a 37.32-fold increase in specific activity, and 31% recovery. PSA showed a molecular weight of approximately 57 kDa by SDS-PAGE. The N-terminal amino acid sequence of the first 7 amino acids was DQAGKSP. This enzyme exhibited maximum activity at pH 8.0 and 55°C, performed stably over a broad range of pH 5.0 ~ 12.0, but rapidly lost activity above 50°C. Both potato starch and Ca²⁺ ions have a protective effect on the thermal stability of PSA. The enzyme activity was inhibited by Hg²⁺, Mn²⁺, Cd²⁺, Cu²⁺, and Co²⁺, and enhanced by Ba²⁺. PSA belonged to the EDTA-sensitive α-amylase. The purified enzyme showed high stability towards surfactants (Tween 20, Tween 80 and Triton X-100), and oxidizing agents, such as sodium per borate and H₂O₂. In addition, PSA showed excellent compatibility with a wide range of commercial solid and liquid detergents at 30°C, suggesting potential application in the detergent industry. Maltotetraose was the specific end product obtained after hydrolysis of starch by the enzyme for an extended period of time, and was not further degraded.

Keywords: G4-α-amylase, PSA, *P. stutzeri* AS22, purification, biochemical characterization, detergent industry

1. Introduction

Amylases are starch degrading enzymes classified as glycoside hydrolases (GHs), which act upon the α-(1,4) and/or α-(1,6) glucosidic linkages of starch, glycogen and related polysaccharides. Alpha-amylases (EC 3.2.1.1, 1,4-α-D-glucan glucohydrolase), classified in family 13 of glycosyl hydrolases, are endo-acting amylases, which randomly cleave α-(1,4) glycosidic bonds of starch polymers internally, resulting in oligosaccharides with varying lengths [1]. This class of industrial enzymes constitutes approximately 30% of the world’s enzyme market that occupies second place after proteases [2,3], covering many industrial processes [4], such as sugar, textile, distilling, baking industries, preparation of digestive aids, production of starch syrups, and enzymatic detergent formulation [5-7]. In laundering, the demand for novel amylases that are effective at lower temperature and stable under detergent conditions including alkaline pH is increasing day by day to preserve the fabric softness when washing clothes [8].

Among amylolytic enzymes, which are produced by a wide number of microorganisms [9], such as *Bacillus* and *Pseudomonas* sp., maltotetraose-forming amylase (EC 3.2.1.60 exomaltotetraohydrolase), which hydrolyzes α-1,4-glucosidic linkages in amylaceous polysaccharides to specifically remove maltotetraose residues from the non-reducing chain ends, has been reported in *Pseudomonas stutzeri* [10,11], *Pseudomonas stutzeri* NRRL B-3389 [12,13], *Pseudomonas stutzeri* strain 7193 [15], *Pseudomonas* sp. IMD353 [16], *Pseudomonas saccharophila* [17], *Micrococcus* sp. [18], *Bacillus circulans* [19], *Bacillus* sp. GM8901 [20], and *Bacillus halodurans* MS-2-5 [21].

In the present study, we report the purification and characterization of an extracellular G4-α-amylase produced...
2. Materials and Methods

2.1. Isolation and identification of amylase producing strain
A total of 20 isolates from the soil of detergent industry SODET in Sfax City-Tunisia were screened for amylase production. The screening of amylolytic microorganisms was carried out on nutrient agar containing soluble starch (1% w/v). After incubation at 37°C for 24 h, the plates were flooded with iodine solution at room temperature. Colonies exhibiting halo starch hydrolyzing activity were picked up. The strain AS22 that produced high level of amylase was used for further studies. It was identified as Pseudomonas stutzeri, according to the methods described in Bergey’s Manual of Systematic Bacteriology [22], and on the basis of the 16S rDNA sequence analysis. P. stutzeri AS22 strain was routinely maintained on Luria-Bertani (LB) agar plates, and conserved in LB medium added to 30% glycerol at -80°C.

2.2. Production of P. stutzeri G4-α-amylase
Inocula were routinely grown in Luria-Bertani (LB) broth medium composed of (g/L): peptone 10.0, yeast extract 5.0, and NaCl 5.0 [23]. The growth medium used for α-amylase production by P. stutzeri AS22 strain was composed of (g/L): potato starch 10.0, yeast extract 5.0, MgSO₄ 0.1, K₂HPO₄ 1.4, KH₃PO₄ 0.7, and NaCl 0.5. The medium was adjusted to pH 8.0. Media were autoclaved at 121°C for 20 min.

Cultivations were conducted in 25 mL of medium in 250 mL conical flasks. Incubations were carried out at 30°C, in a rotary shaker, with stirring at 200 rpm for 24 h. The cultures were centrifuged, and the cell-free supernatants were used as a crude enzyme preparation.

2.3. α-amylase activity assay
α-Amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The reaction mixture, containing 0.5 mL of appropriately diluted enzyme and 0.5 mL of 1% (w/v) potato starch (Sigma) in 100 mM Tris-HCl buffer (pH 8.0), was incubated under optimal conditions of enzyme action. The amount of liberated reducing sugar was determined by the dinitrosalicylic acid (DNS) method [24]. One unit of amylase activity was defined as the amount of enzyme that released 1 µmol of reducing end groups per minute. D-Glucose was used to construct a standard curve.

2.4. Purification of P. stutzeri G4-α-amylase
All purification procedures were carried out at 4°C.

2.4.1. Anion exchange chromatography
The crude enzyme preparation was applied to a Sepharose mono-Q column (2 cm × 25 cm), previously equilibrated with buffer A (25 mM Tris-HCl, pH 8.0). After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0 ~ 0.5 M in the equilibrating buffer. Fractions of 5 mL were collected at a flow rate of 94 mL/h, and analyzed for amylase activity and protein.

Fractions (90 ~ 97) showing α-amylase activities were pooled, and applied to a stirred ultrafiltration cell (Millipore 8400), using 10 kDa MW cut-off membrane (PBGC membrane, millipore).

2.4.2. Sephadex G-100 chromatography
The concentrated enzyme preparation was applied to Sephadex G-100 column (2.6 cm × 6 cm), previously equilibrated with buffer A containing 0.5% Triton X-100. For eluting the amylase from the column, a starch solution (1%) (In the same buffer) was applied. Enzyme fractions of 3 mL were eluted at a flow rate of 20 mL/h. Protein contents and α-amylase activity were determined.

2.4.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-PAGE was carried out for the determination of purity and molecular weight of the α-amylase, as described by Laemmli [25] (1970), using 5% (w/v) stacking and 15% (w/v) separating gels. Samples were prepared by mixing the purified enzyme at 1:5 (v/v) ratio with distilled water containing 10 mM Tris-HCl pH 8.0, 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. Samples were heated at 100°C for 5 min, before electrophoresis. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R250 in 45% ethanol-10% acetic acid, and destained with 5% ethanol-7.5% acetic acid. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit (Amersham Biosciences), as markers consisting of: phosphorylase b (97,000 Da), albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-P dehydrogenase (36,000 Da), bovine trypsinogen (24,000 Da), trypsin inhibitor (20,100 Da) and α-lactalbumin (14,200 Da).

2.4.4. Native-PAGE and zymography
Native-PAGE was performed, according to the procedure