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SHORT COMMUNICATION

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A thermostable glucoamylase from a thermophilic *Bacillus* sp.: characterization and thermostability

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Abstract A thermostable glucoamylase (GA) showed optimum activity at 70°C and pH 5.0. It was highly stable at pH 7.0. The half-life of the enzyme at pH 7.0 was 13, 8, and 3 h 40 min at 60, 65, and 70°C respectively. The residual activity of the enzyme sample incubated at 5 psi (110°C) for 30 min was about 32% of the control set (incubated at 4°C), while no activity was observed at 10 and 15 psi. The thermostability of the enzyme was enhanced twofold in the presence of 0.5% (w/v) starch at 5 psi. Thin-layer chromatography indicated that this enzyme is a GA.

Keywords Glucoamylase: partial purification · Thermostability

Introduction

Glucoamylase ($\alpha 1 \rightarrow 4$ glucan glucohydrolase, EC 3.2.1.3) catalyzes the release of glucose from the non-reducing end of oligo- and polysaccharide chains [1]. Along with α-amylases, glucoamylases (GAs) are used extensively in the industrial hydrolysis of starch. At the standard reaction temperature of >60°C (pH 5.0), however, the practical utility of GAs is somewhat restricted due to irreversible inactivation at high temperature [2]. Therefore, an economical source of active and more thermostable GA is industrially desirable.

Extremophiles have developed a variety of molecular strategies in order to survive under harsh conditions [3]. Generally, enzymes from thermophiles are more thermostable than their mesophilic counterparts. Thus, attempts have been made to isolate a thermophilic microorganism producing thermostable GA. In the present investigation, we report the isolation, characterization, and thermostability studies of a thermostable GA produced by a thermophilic microorganism.

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Materials and methods

Microorganism and culture conditions

The bacterial strain used in this study was isolated from 68 thermophilic amylase-producing microorganisms obtained after thorough screening of samples collected from the thermal springs of Manikaran, Himachal Pradesh, India. The strain was grown on 2% (w/v) Luria Bertani broth (pH 7.0) under stationary and shaking conditions (150 rpm), inoculated at 2% (v/v) and incubated for 17–20 h at 60–65°C. The supernatant was used for the estimation of GA activity.

Enzyme assay

Glucoamylase activity was determined by the DNSA (3,5-dinitrosalicylic acid) method with slight modifications. The enzyme assay was carried out at 70°C and pH 5.0 (acetate buffer) or pH 7.0 (phosphate buffer) [4]. One unit of enzyme activity is defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute under the assay conditions.

Partial purification of enzyme

The cell culture (500 ml) was centrifuged at 5,000×*g* for 15 min. Ammonium sulfate (65% final concentration) was added to the supernatant. After stirring it for 4 h at 4°C, the precipitate was collected by centrifugation at 12,000×*g*. The pellet was dissolved in 10 ml of 50 mM Tris–HCl buffer (pH 8.0) and 7 ml was loaded on a Sephadex G-150 column (1×70 cm, Sigma superfine grade). Fractions of 4 ml were collected and assayed for enzyme activity. The fractions having activity were

pooled (28 ml) and loaded onto a Q-Sepharose column (2.4×4 cm^2, Sigma) pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The column was washed with five column volumes of the same buffer. The bound protein was eluted stepwise with 50 mM Tris–HCl buffer (pH 8.0) containing 0.15 m NaCl (two column volumes) followed by 0.25 m NaCl (three column volumes). The fractions having enzyme activity were pooled, dialyzed, and concentrated. Protein concentration was estimated after each purification step by the method of Lowry (Table 1).

Thermostability of the enzyme

The enzyme was incubated at different temperatures (60–90°C) for 30 min at pH 5.0 or 7.0 to examine its thermostability. In order to calculate the half-life of the enzyme, samples containing the enzyme were incubated for different time points at 60, 65 and 70°C. In a separate set of experiments, the partially purified enzyme was incubated at 5, 10 and 15 psi (at 110, 115 and 121°C, respectively) in an autoclave at pH 7.0 for 30 min. After cooling the samples at 4°C for 30 min, the enzyme was assayed for activity. The non-treated enzyme served as control (100%).

In order to investigate the effect of starch on enzyme stability, the enzyme was incubated in the presence of different concentrations of starch (0.25–5.0%) at 5 psi for 30 min and then cooled at 4°C for 30 min. As the starch included in the samples might have been degraded during the incubation process, the enzyme after cooling was incubated again at 70°C for 30 min (to determine remaining enzyme activity after incubation at 5 psi) followed by standard enzyme assay. The difference berween the amount of starch degraded before (control) and after incubation at 70°C (experimental) reflects the level of enzyme activity in each sample.

End product(s) analysis/mode of action

Enzyme was incubated with 0.25% (w/v) substrates (starch, maltose) soluble in 50 mM phosphate buffer, pH 5.0 for various time periods at 70°C. Hydrolysates (5 µl each) and 2% standard sugars (glucose and maltose) mixed at a ration of 1:1 (5 µl each) were spotted on Silica gel-60 plates. The product(s) produced following the enzymatic hydrolysis of starch were identified by thin-layer chromatography (TLC) using the solvent system of *n* butanol:ethanol:water (5:3:2). Spots were revealed by spraying 50% (v/v) sulfuric acid and heating for 10 min at 110°C.

Results and discussion

A bacterial strain producing a thermostable GA was isolated. According to Bergey's manual of systematic bacteriology [5], the strain was identified as *Bacillus* sp. (named *Bacillus* sp. strain J38) based on characteristics such as gram-positive; aerobic; catalase-positive; motile spore-forming; rod shaped. Production of GA was maximum at 65°C and pH 7.0 and was higher (13.4 ± 2.9 U/ml) under stationary conditions after 17–20 h incubation than under shaking conditions (8.2 U/ml). Luria broth, supplemented with 0.5% (w/v) soluble starch, yielded good growth as well as highest enzyme activity (21.6 U/ml).

The enzyme was partially purified to rule out the possibility of synergistic effect of other amylases in crude preparation. The partially purified enzyme, which appeared as a few bands on SDS-PAGE, was used for further studies. The enzyme hydrolyzed maltose and starch to give glucose as the sole end product as revealed by TLC. This clearly indicates the saccharogenic nature of the enzyme. Activity was measured over a broad pH range 4.0–8.0 (at 70°C) with optimal activity at pH 5.0 and maximum stability at pH 7.0 (Fig. 1). The enzyme was active over a broad temperature range (60–80°C) (Fig. 2). At pH 7.0, it was stable up to 80°C for 30 min, but at the same temperature and pH 5.0, a 40% loss of enzyme activity was observed. After a 10-min incubation at pH 7.0 in a boiling water bath, 36% of the enzyme activity was retained , while only 11% activity was retained at pH 5.0. The half-life of the enzyme was 13 h, 8 h and 3 h & 40 min at 60, 65 and 70°C, respectively, at pH 7.0 (Fig. 3). The high-temperature optima (70°C at pH 5.0 and 80°C at pH 7.0) of the strain J38 GA confirmed its uniqueness.The temperature optima of other reported GAs are generally in the range of 40–60°C [6, 7], except for that of bacterium *Clostridium*, which has optima of 70–75°C [8], and *Scylalidium thermophilum* ,with temperature optima of 70°C [9]. A GA from the thermophilic mold *T. indicae-seudaticae* had optimal activity at 60°C and pH 7.0, with a $t_{1/2}$ of 12 h at 60°C and 7 h at 80°C [10]. Both the optimal activity parameters (thermal stability and activity as a function of

Table 1 Purification profile of *Bacillus* sp. strain J38 GA

Purification step	Total protein (mg)	Total enzyme (U)	Specific activity (U/mg protein)	Purification (-fold)	Yield (%)
Supernatant	3,153	10,500	3.33	1.0	100
Ammonium sulphate precipitation	952.5	9,240	9.7	2.91	88
Gel filtration	340.5	6,300	18.5	5.6	60
Q-sepharose	77.0	2,625	34.0	10.2	25

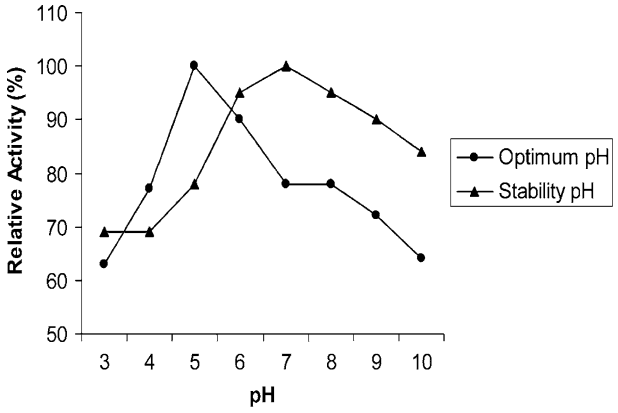

Fig. 1 pH activity and stability profile of *Bacillus* sp. strain J38 glucoamylase (GA) at 70°C. Enzyme activity was measured at different pH values (4–10). In order to determine stability, the enzyme was incubated at different pH values (3–10) for 24 h followed by measuring enzyme activity at pH 5.0. The enzyme activity at pH 5.0 (20.8 U/ml) was taken as 100%

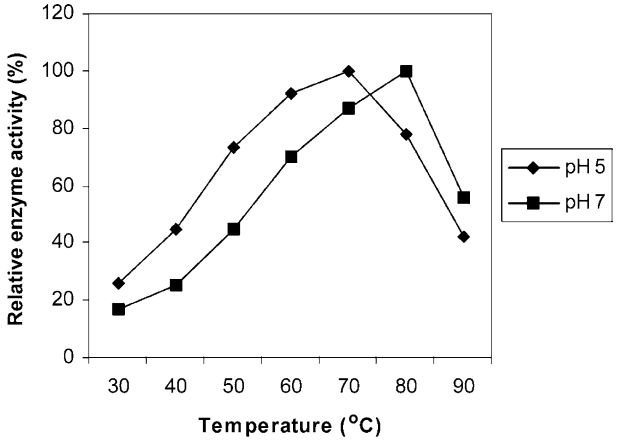

Fig. 2 Temperature activity profile of *Bacillus* sp. strain J38 GA at pH 5.0 and pH 7.0. Enzyme activity was measured over a range of temperatures (30–90°C). The enzyme activities at 70°C, pH 5.0, and 80°C, pH 7.0, were taken as 100% (20.3 U/ml)

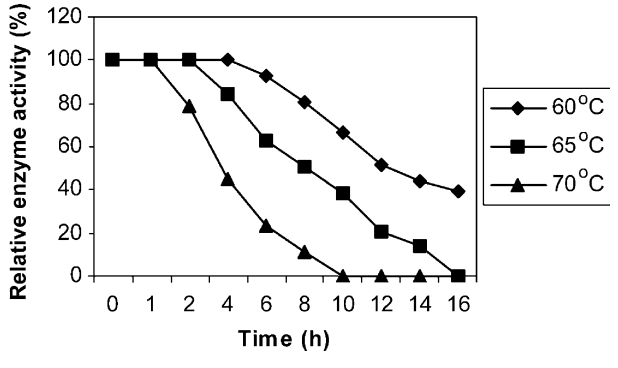

Fig. 3 Half-life of the *Bacillus* sp. strain J38 GA at different temperatures (60, 65 and 70°C) and pH 7.0. The activity of a non-incuabted sample (22.1 U/ml) was taken as 100% (20.3 U/ml)

temperature) and the extreme conditions (retention of conformation at high temperature), under which the native quaternary structure of the enzyme is maintained demonstrate the substantial thermal stability of this GA. The GA of *Clostridium* retains 50% activity at 85°C for 1 h [8] while the enzyme of strain J38 retains 100% activity at 80°C for 1 h. The optimum pH was found to be similar to those of other GA reported [6]. By contrast, the enzyme was more stable at pH 7.0 than at pH 5.0. In order to exploit the synergism required in starch hydrolysis, the temperature and pH optima of α-amylase and GAs need to be compatible. However, while the pH optima of most GAs are at or near pH 4, α-amylases active and stable at this low pH are rare. The GA reported here is optimally active at 70°C (pH 5.0) and 80°C (pH 7.0), conditions under which raw-starch-digesting α-amylases is also active [11]. Therefore, thet GA of strain J38 is a good candidate for synergism in starch degradation.

Enzyme activity was analyzed in the presence of 10 mM metal ions. Activity was completely inhibited by K^+, Cu^{2+} and Hg^{2+} but stimulated by Mg^{2+} (142%), Co^{2+} (131%) and Fe^{2+} (126%). In the presence of Ca^{2+}, a 60% loss in enzyme activity was measured. The loss of enzyme activity in the presence of $HgCl_2$, which is a non-specific thiol-group inhibitor, suggests cysteine residues participate in conferring activity.

The residual activity of the enzyme sample incubated at 5 psi for 30 min was about 32% of the control (100% activity), while the enzyme sample incubated at 10 and 15 psi did not show any activity. The presence of starch had a profound effect on enzyme stability at higher temperature; stability was enhanced nearly twofold in the presence of 2.5% starch. No further stabilization was observed at a higher concentration of starch. Enhanced thermostability in the presence of amylase substrate (starch) has been reported previously [6]. Nielsen et al. [12] described a significantly improved thermostability of GA from *T. emersonii* in 30% (w/v) glucose. It was proposed that binding of substrate to the active site may increase enzyme stability.

In conclusion, the GA from *Bacillus* sp. strain J38 is a novel biocatalyst that may find potential applications in starch processing as it allows stach hydrolysis without gelatinization.

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