Structural and biochemical properties of lichenase from *Clostridium thermocellum*

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Abstract The recombinant enzyme lichenase of size 30 kDa was over-expressed using *E. coli* cells and purified by immobilized metal ion affinity chromatography (IMAC) and size exclusion chromatography. The enzyme displayed high activity towards lichenan and β -glucan. The enzyme showed no activity towards carboxymethyl cellulose, laminarin, galactomannan or glucomannan. Surprisingly, affinity-gel electrophoresis on native-PAGE showed that the enzyme binds only glucomannan and not lichenan or β -glucan or other manno-configured substrates. The enzyme was thermally stable between the temperatures 60°C and 70°C. Presence of Cu^{2+} ions at a concentration of 5 mM enhanced enzyme activity by 10% but higher concentrations of Cu^{2+} (>25 mM) showed a sharp fall in the enzyme activity. Heavy metal ions Ni^{2+} , Co^{2+} and Zn^{2+} did not affect the activity of the enzyme at low concentrations (0–10 mM) but at higher concentrations (>10 mM), caused a decrease in the enzyme activity. The crystals of lichenase were produced and the 3-dimensional structure of native form of enzyme was previously solved at 1.50 Å. Lichenase displayed

(β/α)₈-fold a common fold among many glycoside hydrolase families. A cleft was identified that represented the probable location of active site.

Keywords Lichenase · Glycoside hydrolase · *Clostridium thermocellum* · Cellulase

Introduction

Glycoside hydrolases have been grouped into 113 families based on the sequence homology (<http://www.cazy.org/>) [1]. A bifunctional cellulase from *Clostridium thermocellum* was shown to contain an N-terminal family 26 glycoside hydrolase called lichenase (Lic26A) [2] and an internal family 5 glycoside hydrolase (GH5) [3] catalytic domains and a C-terminal family 11 carbohydrate-binding module (CBM11) [4, 5]. It was demonstrated that the carbohydrate-binding module (CBM11) of bifunctional cellulase binds to ligands that are substrates for both Lic26A and GH5 catalytic modules [4]. The crystal structure of lichenase has been determined and interestingly it has been shown that the enzyme displays high activity towards lichenan and the mechanism of binding and catalysis of lichenan was proposed [2]. The optimum temperature and pH of lichenase enzyme was determined and was characterized for urea stability [6]. The enzyme gave maximum activity in the temperature range 60–80°C. The family 26 of glycoside hydrolases generally contains mannanases but the enzyme Lic26A did not show any activity towards manno-configured substrates such as mannan, galactomannan or glucomannan. Surprisingly, the enzyme was active with lichenan and β -glucan, so it was named lichenase, Lic26A [2, 6]. In the present paper we report the biochemical and structural characteristics of recombinant lichenase enzyme obtained from *Clostridium thermocellum*.

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Materials and methods

Over-expression, purification and activity assay of lichenase

The lichenase was over-expressed using *E. coli* BL-21 cells containing the recombinant plasmid following the methods described earlier [6]. The over-expressed enzyme was soluble and upon sonication comes out in cell-free extract as soluble fraction. The protein was purified by immobilized metal ion affinity chromatography (IMAC) as described previously [7]. For crystallization, C_LLic26A was further purified by size exclusion chromatography [2]. The purified enzyme was analyzed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [8]. The pure protein as judged by SDS-PAGE was concentrated to 20 mg/ml. The enzyme assay was carried out by incubating the enzyme with substrate for 10 min at 70°C. The reaction mixture (100 µl) contained 10 µl of enzyme (0.24 mg/ml) and 0.2 % final concentration of lichenan in 50 mM sodium phosphate buffer pH 7.0. The 100 µl mixture was analyzed for the release of reducing sugar using the Nelson [9] and Somogyi [10] method.

Thermal stability of lichenase

The thermal stability of lichenase was determined by incubating the 50 µl enzyme (0.24 mg/ml) for 15 min at various temperatures ranging from 30°C to 90°C. Aliquots (10 µl) of the enzyme were withdrawn and assayed in 100 µl reaction mixture containing 0.2% lichenan in 50 mM sodium phosphate buffer pH 7.0 and incubated at 70°C for 10 min. The released reducing sugar was analysed by methods described earlier.

Effect of heavy metals on lichenase activity

The effect of certain heavy metal salts, such as CuSO₄, NiSO₄, CoCl₂, ZnSO₄ and HgCl₂, on the activity of lichenase was studied varying their concentrations from 0 to 50 mM. The 100 µl reaction mixture contained 10 µl enzyme (0.24 mg/ml), lichenan (0.2%) in 50 mM sodium phosphate buffer (pH 7.0) with varying concentrations of CuSO₄, NiSO₄, CoCl₂, ZnSO₄ and HgCl₂. The reaction mixture was incubated at 70°C for 10 min. The enzyme activity was calculated as described earlier.

Affinity electrophoresis of lichenase

The binding ability of lichenase to soluble polysaccharides such as lichenan, carboxymethyl cellulose (CMC) and glucomannan were evaluated by native affinity gel electropho-

resis. Native polyacrylamide gels were prepared consisting of 7.5% (w/v) acrylamide in 25 mM Tris/250 mM glycine buffer [5]. The substrates (1.0%) were added to the polyacrylamide mixture before polymerization. Approximately 10 µg of lichenase and bovine serum albumin (BSA) (as non-interacting negative control) were loaded to the gels and subjected to electrophoresis at 2mA/lane for approximately 2 h at room temperature. A control gel was also run with lichenase and BSA without the substrate in gel.

Results and discussion

Thermal stability of lichenase

The catalytic module displayed thermostable properties exhibiting a profile where the enzyme was highly stable at 70°C. The activity of the enzyme decreased sharply by more than 90% at a temperature of 80°C when incubated for 15 min, whereas only 20% of enzyme activity was lost within 15 min, when incubated at temperature 60°C (Fig. 1). The enzyme is thermally stable between the temperatures 60°C and 70°C, which is in accordance with its thermophilic origin.

Effect of heavy metal salts on lichenase activity

A very low concentration range (0–5 mM) Cu²⁺ enhanced lichenase activity by 10% (Fig. 2A) like Ca, Mg and Mn salts [6]. Similar results were also reported for a β-1,3-xylanase belonging to GH26 family [11]. However, higher concentrations of Cu²⁺ (>25 mM) resulted in a sharp fall in lichenase activity and the activity dropped to 0 % at 50 mM. Some of the heavy metal ions Ni²⁺, Co²⁺ and Zn²⁺

Fig. 1 Thermal stability of lichenase

Fig. 2 Effect of heavy metal salts (A) CuSO₄ (B) NiSO₄ (C) CoCl₂ and (D) ZnSO₄ on lichenase activity

Fig. 3 Affinity gel-electrophoresis of lichenase with glucomannan in 7.5% native-PAGE. Lane 1: bovine serum albumin (BSA), Lane 2: Lic26A without 1% glucomannan and Lane 3: Lic26A with 1% glucomannan

did not affect the activity of enzyme at low concentration range (0–10 mM) but at higher concentrations (>10 mM) the enzyme activity decreased (Figs. 2B, 2C and 2D). The

Fig. 4 Crystals of seleno-methionine derivative of lichenase

enzyme activity of lichenase was completely inhibited by Hg²⁺ salt (data not shown).

Affinity electrophoresis of lichenase

The family 26 glycoside hydrolase contains predominantly mannanases, but lichenase Lic26A did not show any

Fig. 5 (A) A representation of lichenase (Lic26A) enzyme surface. The cleft indicated by the arrow represents a potential substrate-binding site and is the probable location of the active site. (B) Illustration of Lic26A displaying a common $(\beta/\alpha)_8$ fold among many glycoside hydrolase families.

activity towards mannan, galactomannan or glucomannan. Surprisingly, the enzyme lichenase, Lic26A showed activity towards lichenan and β -glucan. It showed no activity towards other soluble polysaccharides such as carboxymethyl cellulose or laminarin [6]. Interestingly, analysis by affinity electrophoresis on native-PAGE showed that lichenase binds glucomannan but does not show any enzyme activity towards this substrate as mentioned earlier. The enzyme movement in the gel was retarded in the presence of 0.1% glucomannan (Fig. 3). These data show that lichenase from *Clostridium thermocellum* has narrower substrate specificity as compared to other similar glycoside hydrolases of the same family.

Structural properties of lichenase

The crystals of lichenase, Lic26A were produced (Fig. 4) as described earlier [2] and the 3-dimensional structure of the native form of Lic26A was solved at 1.50Å. Figure 5A was prepared with PyMOL (<http://pymol.sourceforge.net>) that represents the surface of Lic26A in which the cleft indicated by the arrow represents a potential substrate-binding site and is also the probable location of the active site. Figure 5B was generated using MOLSCRIPT [12] showing that the 3-dimensional structure of Lic26A displays a $(\beta/\alpha)_8$ fold, a common fold among many glycoside hydrolase families.

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