

Some Properties of Crude Carboxymethyl Cellulase of *Aspergillus niger* Z10 Wild-Type Strain

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Abstract: A carboxymethyl cellulase enzyme was prepared from a wild type strain of *Aspergillus niger* Z10. Analyses of the enzyme preparation by SDS-PAGE revealed two protein bands showing cellulolytic activity. The molecular weight of these bands was estimated to be around 83,000 and 50,000. The optimum temperature of the enzyme was observed to be around 40 °C. It was found that the enzyme's activity has a broad pH range between 3 to 9 and 41.2% of the original activity was retained after heat treatment at 90 °C for 15 min.

Key Words: *Aspergillus niger*, Carboxymethyl cellulase (CMCase)

Aspergillus niger Z10 Yaban Tip Suşundan Elde Edilen Ham Karboksümetil Selülaz'ın Bazı Özellikleri

Özet: *Aspergillus niger* Z10 yaban tip suşundan karboksümetil selülaz enzimi hazırlanmıştır. Enzim preparasyonunun SDS-PAGE analizi sonucunda, jel üzerinde selülitik aktivite gösteren iki protein bandı saptanmıştır. Bu bandların moleküler ağırlıkları yaklaşık olarak sırasıyla 83000 ve 50000 olarak hesaplanmıştır. Enzimin optimum sıcaklığının 40 °C civarında olduğu gözlenmiştir. Enzimin aktivite gösterdiği pH aralığının ise 3 ile 9 arasında olduğu bulunmuş ve enzim 90 °C'de 15 dakika tutulduğunda, orijinal aktivitesinin % 41.2'nin devam ettiği kaydedilmiştir.

Anahtar Sözcükler: *Aspergillus niger*, Karboksümetil selülaz (CMCaz)

Introduction

Cellulose is the most abundant and renewable biopolymer on Earth. Cellulose has been used by man for centuries; however, its enormous potential as a renewable source of energy was recognised only after cellulose degrading enzymes or "cellulases" had been identified (1).

The biotechnology of cellulase and hemicellulases began in the early 1980s, first in animal feed and then in food applications (2). Subsequently, these enzymes were

used in the textile, laundry and pulp and paper industries. Today, these enzymes account for approximately 20% of the world enzyme market, mostly from *Trichoderma* and *Aspergillus*. Filamentous fungi, *Aspergillus* and *Trichoderma* species in particular, are well known and efficient producers of plant cell wall-degrading systems. These organisms consist of three classes of enzymes endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β -glucosidases (EC 3.2.1.21). Members of all these classes are necessary to degrade cellulose (3,4).

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Cellulosic substrates hydrolysed by only one type of cellulase are categorised as follows: acid-swollen cellulose, carboxymethyl cellulose (CMC), cellulose azure, and trinitrophenyl Cm-cellulose are hydrolysed by endoglucanases. Methylumbelliferyl- β -D-cellobiose (MUC), and p-nitrophenyl- β -D-cellobioside (pNPC) are used as substrates for the determination of exoglucanase activity, and methylumbelliferyl- β -D-glycopyranoside (MUG) and p-nitrophenyl- β -D-glycopyranoside (pNPG) are cleaved by β -glycosidases (5).

In the current study, some properties such as the molecular weight, optimum pH, temperature, and heat stability of carboxymethyl cellulase (CMCase) of *Aspergillus niger* Z10 natural isolate are reported with a view to identify potential sources of industrial enzymes.

Materials and Methods

Fungal strain and culture conditions

A. niger Z10 wild-type strain was used as an enzyme source. Cultivation was carried out in Czapek Dox liquid medium containing CMC (1%) as a sole carbon source. For growth of the organism, a 1 L Erlenmeyer flask containing 250 ml of growth medium was kept at 28 °C on an orbital shaker set at 250 rev.min⁻¹ for 5 days. Mycelia was removed by filtration and the filtrate was used for crude enzyme preparation.

Crude enzyme preparation

To the culture filtrate (250 ml), ethanol previously chilled to -20 °C was added dropwise at 4 °C with continuous stirring to the final concentration of 75% and the solution was left at -20 °C for 24 h. The resultant precipitate was collected by centrifugation and dissolved in 30 ml of phosphate buffer (50 mM, pH 5.0) and was dialysed against the same buffer (pH 5.0) overnight at 4 °C (6).

Enzyme assay

CMCase assay was investigated for CMC-saccharifying activity by incubating 0.5 ml of enzyme solution with 0.5 ml of CMC (1%) in phosphate buffer (50 mM, pH 5.0) for 30 min at 37 °C. Released sugars were estimated by Dinitrosalicylic acid (DNS) reagent (7).

Optimum temperature, pH and heat stability

In all the determinations, CMCase activity was measured using CMC as a substrate. For the estimation of

optimum temperature, pH and heat stability, activity was determined by carrying out the above standard assay at several temperatures or pH values.

For the determination of the optimum temperature of the enzyme, the enzyme was incubated with substrates for 30 min at various temperatures between 30 and 90 °C. For the determination of optimum pH, the enzyme was mixed with substrates at different pH levels (pH 3.0 to 9.0). These substrates were prepared in two buffer solutions: citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na₂HPO₄, pH 3.0 to 7.0), and Tris buffer (0.08 M Tris, 0.1 M HCl, pH 7.5 to 9.0). For temperature stability, the enzyme was incubated in the standard buffer at 40, 50, 60, 70, 80 and 90 °C for 15 min and reaction tubes were placed in ice. Then the activity was assayed by DNS method under the standard conditions outlined in our previous paper (8).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

For the determination of homogeneity and molecular weight, the enzyme preparations and known molecular weight markers were subjected to electrophoresis by the method of Bollag and Edelstein (9) with the use of 10% acrylamide gel; 0.2% CMC was incorporated into the separating gel prior to the addition of ammonium persulphate and polymerisation. After electrophoresis, the gel was stained with Coomassie Blue R dye in methanol-acetic acid-water solution (4:1:5, by volume) for 1 h and destained in the same solution without dye.

For the activity staining of CMCase activity, SDS was removed by washing the gel at room temperature in Solution A (sodium phosphate buffer, pH 7.2, containing isopropanol 40%) for 1 h and Solution B (sodium phosphate buffer, pH 7.2) for 1 h respectively. Renaturation of the enzyme proteins was carried out by leaving the gel in Solution C (sodium phosphate buffer, pH 7.2, containing 5 mM β -mercaptoethanol and 1 mM EDTA) at 4 °C overnight. The gel was then transferred onto a glass plate, sealed in a film, and incubated at 37 °C for 4-5 h. The gel was stained in a solution of 1% Congo Red for 30 min, and destained in 1 M NaCl for 15 min. Clear bands indicated the presence of CMCase activity (10-12).

Results

Molecular weight

Molecular weight was determined by SDS-PAGE as described in the Materials and Methods above. Analyses of the enzyme by SDS-PAGE revealed two bands which show cellulolytic activity in the gel (Figure 1 B). The molecular weight of these bands was estimated to be about 83,000 (Band 1) and 50,000 (Band 2) respectively (Figure 1 A).

Optimum temperature of the enzyme

For estimation of the optimum temperature of the enzyme, the activity was determined by carrying out the assay at several temperatures between 30° and 90 °C. The optimum temperature was observed to be around 40 °C (Figure 2).

pH optimum of the enzyme

The pH optima were determined in two buffer systems (citrate-phosphate buffer and Tris buffer). It was observed that the enzyme's activity has a broad pH range between 3.0 and 9.0. Two major activity peaks were obtained at pH 4.5 and 7.5 (Figure 3).

Heat stability

The thermostability of the enzyme was studied by heating the enzyme at different temperatures (40-90 °C) for 15 min. As a result, 41.2% of the original activity was retained after heat treatment at 90 °C for 15 min (Figure 4).

Discussion

CMCase was obtained from *A. niger* Z10 wild-type strain in a liquid Czapek Dox medium containing carboxymethyl cellulose (1%) as the sole carbon source. The enzyme was extracted from culture supernatant by the ethanol precipitation method. The crude enzyme preparation was subjected to SDS-PAGE (containing 0.2% CMC) to determine the homogeneity and molecular weight of the enzyme. During the electrophoresis of the enzyme, two bands showing cellulolytic activity were detected (Figure 1 B). The molecular weights of these proteins were calculated to be about 83,000 and 50,000 respectively (Figure 1 A). It was reported that these proteins may be isoenzymes or the different subunits of the same enzyme proteins. A cellulolytic enzyme from *A.*

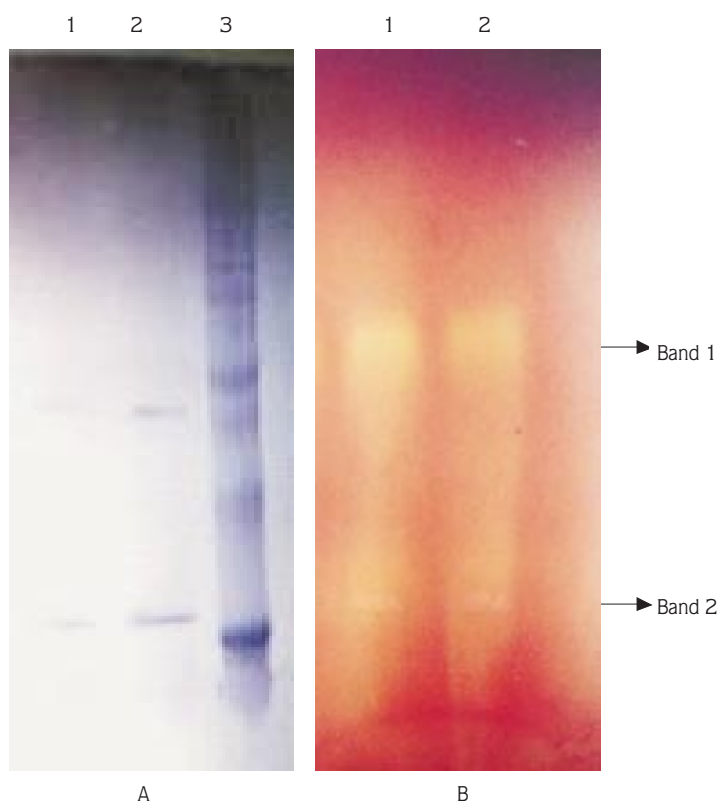


Figure 1. Separation (A) and activity pattern (B) of *A. niger* Z10 CMCase in SDS-Polyacrylamide gel. Electrophoresis was carried out in SDS-Polyacrylamide gel containing 0.2% CMC. Gel was stained as described under the Materials and Methods. Figure 1A) Lanes 1 and 2 contains crude CMCase from *A. niger* Z10. Lane 3 contains the molecular weight markers: 205,000, 116,000, 97,400, 66,000, 45,000 and 29,000. Figure 1B) Line 1 and 2 crude CMCase from *A. niger* Z10. The zones appeared after staining gel with Congo Red dye.

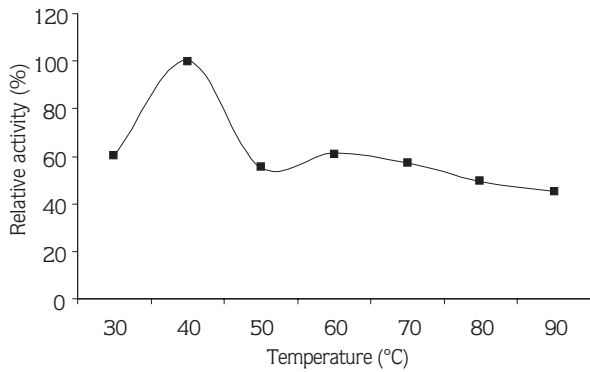


Figure 2. The optimal temperature range of CMCase of *A. niger* Z10.

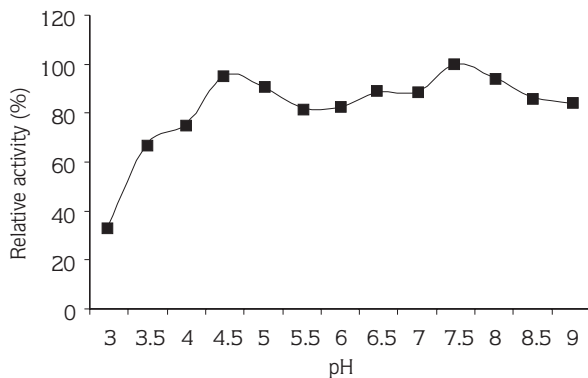


Figure 3. The optimal pH range of CMCase of *A. niger* Z10. Two buffer systems were used to determine the optimal pH range of the enzyme: citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na₂HPO₄, pH 2.6 to 7.0) and Tris buffer (0.08 M Tris, 0.1 M HCl, pH 7.5 to 8.9).

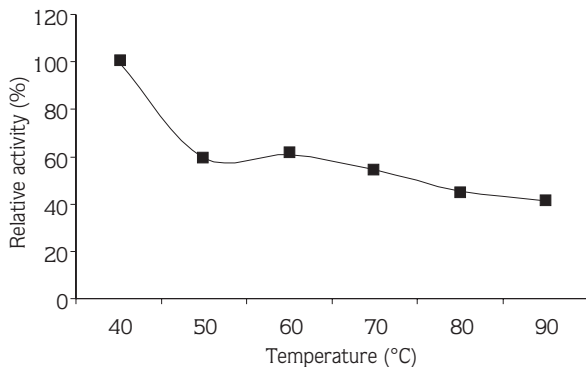


Figure 4. Heat stability of CMCase of *Aspergillus niger* Z10.

niger was separated as a homogeneous entity in SDS-PAGE, but showed one major and two minor bands in disc gel electrophoresis (13). In the same paper, it was also reported that no carbohydrate was associated with

the protein and the enzyme was rich in acidic and aromatic amino acids. According to other research, two endoglucanase containing fractions were separated from *A. niger* (14). These enzymes possessed no ability to bind to or hydrolyse insoluble microcrystalline cellulose (Avicel), but were active towards soluble carboxymethyl cellulose.

The optimum temperature of the enzyme was found to be around 40 °C. This value is lower than that of commercial cellulase preparation (Deerland cellulase 4000) from *A. niger* (15). Deerland cellulase 4000 is an enzyme preparation derived from *A. niger* and has high cellulase activity together with β-glucosidase and hemicellulase. The optimum temperature of this cellulase was reported to be 60 °C. However, our enzyme preparation was thermostable and stable up to 90 °C (Figure 4). It was reported that a CMCase from *A. niger* RD-2231 isolate was found to be stable at temperatures below 40 °C and CMCase activity decay at 65 °C was 28 times higher than its value at 30 °C (16). On the other hand, endoglucanase from *A. fumigatus* was reported to be stable at 50 °C (17). This comparison shows that the heat stability of CMCase from *A. niger* Z10 is higher than that of the enzymes mentioned above.

It was found that the CMCase activity of *A. niger* Z10 has a broad pH range between 3.0 and 9.0. The enzyme shows two major activity peaks at pH 4.5 and 7.5. This result is probably due to the presence of two isoenzymes or subunits in the enzyme preparation. It was reported that the optimal pH for a CMCase from *A. niger* was found between 6.0 and 7.0 (18), and in another report the optimal pH activity of *A. niger* CMCase was found to be between 4.0 and 4.5 (19). Thus, it was seen that there was no agreement regards enzyme data results. Such different results may appear because of differences within the same genus. In addition, no comparative investigations have been published on the enzymes from these organisms, but the differences appear to be as small as the differences in morphology between the species (20). For example, it was reported that the molecular weight of glucoamylase of *A. niger* varies in the range of 60,000 to 100,000 (20).

Conclusions

Research on cellulase has progressed very rapidly over the last five decades and potential industrial applications

of cellulases have been identified. Major impediments to exploiting the commercial potential of cellulases are the yield, stability and cost of cellulase production. Therefore,

research should also aim at exploiting the commercial potential of existing and new cellulases in nature.

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