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Purification and Some Properties of Endo-1,4-*β*-Glucanases of *Trichoderma harzianum* UzCF-28

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Abstract

This work aimed at isolation, purification and study of biochemical features of cellulolytic enzymes synthesized by *Trichoderma harzianum* UzCF-28 strain. Strain UzCF-28 revealed a high cellulolytic activity during submerged cultivation in the liquid culture on modified Mandels nutrient medium, where wheat straw was used as a source of carbon. As a result of purification by precipitation with ammonium sulfate and further ion exchange chromatography, two isoforms of endo-1,4-*β*-glucanase-EG II and EG III with molecular weight of 135 and 75 kDa respectively were revealed. The pH optimum for EG I and EG III was 4.5, while for EG II—4.7, irrespective of the applied substrates—either CMC or "Whatman filter" paper. Heating up to 40˚C of EG III did not lead to its inactivation, and on the contrary, its activity increased by more than three times comparing to the initial activity of the enzyme, *i.e.* thermostability of EG III among tested enzymes significantly varied.

Keywords

***Trichoderma harzianum*, Enzyme, Endo-1,4-*β*-Glucanase, Purification**

1. Introduction

Cellulose is a polysaccharide composed of hundreds to thousands of d-glucose units linked by *β* (1-4) glycosidic bonds. This means, that two linked d-glucose units are oriented in opposite directions, making rather cellobiose (d-glucosyl-*β*-(1-4)-d-glucose) the repeat unit of cellulose than d-glucose [1]. Cellulases produced by microorganisms are major players in natural processes of cellulose degradation. Aerobic filamentous fungi are widely

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used in production of commercial cellulose [2]. Cellulase, which is produced by fungi and bacteria, can be divided into three major types: endoglucanase (endo-1,4-*β*-D-glucanase, EC 3.2.1.4), cellobiohydrolase (exo-1,4-*β*-D-glucanase, EC 3.2.1.91), and *β*-glucosidase (1,4-*β*-D-glucosidase, EC 3.2.1.21) [3]. Content of cellulase enzyme complexes of filamentous fungi and the activity of their constituent enzymes vary considerably. For example, cellulase complex of fungus *Chrysosporium luckttowense* is represented by endoglucanase and cellobiohydrolase [4]. Cellulase system of *Trichoderma reesei* is represented by 2 cellobiohydrolase, 5 endoglucanase and 2 glucosidase [5], including cellobiohydrolase which makes up to 10% protein mass sheathe [6]. At the same time, complex *Thermoascus aurantiacus* dominates in endo-*β*-1,4-glucanase, which makes up 13.3% [7]. Fungal species have been primarily used commercially for cellulase production because of their capacity to secrete cellulolytic enzymes into their medium, which allows for easy purification and extraction [8]. Isolation of fungal cellulases and study of their physical-chemical properties and kinetics have been conducted by many scientists. As a result, kinetics of endoglucanases, exoglucanases and *β*-glucosidases, synthesized by *Trichoderma harzianum* (L04) [9] has been studied. The factors influencing synthesis of cellulases by *Trichoderma viride* during submerged fermentation have been described in detail [10]. Besides, there were many works on isolation, purification of glucanase, produced by *Trichoderma* sp. with detailed description of its characteristics [11].

This work aimed at isolation, purification and study of biochemical features of cellulolytic enzymes synthesized by *Trichoderma harzianum* UzCF-28 strain, which revealed a high cellulolytic activity during submerged cultivation in the liquid culture on modified Mandels nutrient medium, where wheat straw was used as a source of carbon.

2. Material and Methods

2.1. Object of the Study

Previously isolated and patented UzCF-28 strain of *Trichoderma harzianum* was used as an object of research. This strain was selected as a result of screening among micromycetes of *Aspergillus*, *Trichoderma* and *Penicillium* genera isolated from various plant wastes and soils. Cellulase enzyme complex synthesized during cultivation of this strain showed the highest cellulase activity towards various substrates comparing with other tested micromycetes.

2.2. Nutrient Medium/Submerged Cultivation of the Microorganism

Submerged cultivation of the strainUzCF-28 was carried out on the modified Mandels [12] nutrient medium, where wheat bran (2%) served as a source of carbon. 2 ml of inoculum containing 10^6 conidiums/ml was injected into 100 ml of nutrient medium prepared in 250 ml Erlenmeyer flasks. The fungus was cultivated on a shaker (150 rpm/min) at 30˚C for 72 hours formerly defined as an optimum time when the highest cellulase activity was fixed. The culture liquid of *T. harzianum* UzCF-28 was centrifuged at 3000 g for 30 min and cell free supernatant obtained thereafter served as a crude complex of enzymes for further isolation of endo-1,4-*β*-glucanase.

2.3. Determination of Cellulase Activity

The activity of cellulases of Trichoderma harzianum UzCF-28 was assayed according to the method described by Mandels and Weber [13]. Activity was evaluated by the amount of reducing sugars formed by the action of the enzymes (culture liquid) hydrolyzing preliminary prepared solution of 1% carboxymethyl cellulose (CMC) or a filter paper (Whatman NO. 1) tested as a second comparative substrate at 50˚C for 60 minutes. The reacting solutions consisted of 0.5 ml of the culture liquid (supernatant) and substrate (50 mg of filter paper or 0.5 ml of CMC) submerged in 0.5 ml of 0.05 M acetate buffer at pH 5.5. After hydrolysis, reducing sugars were determined by the method of Somogy-Nelson [14] [15].

2.4. Purification of Endo-1,4-*β*-Glucanase

Precipitation of a crude enzymes complex from the culture liquid was conducted by the method of salting-out with $(NH_4)_2SO_4$ up to 70% saturation [16]. Ion-exchange chromatography on DEAE-Toyopearl^R 650 M [17] was performed to fractionate the enzyme complex. Tests on purity of the enzymes isolated from the culture liq-

uid of *T. harzianum* UzCF-28 was performed by the method of analytical polyacrylamide gel electrophoresis [18] [19]. Electrophoresis was performed on 7.5% polyacrylamide gel.

2.5. Molecular Weight Determination

The molecular weight of the enzymes was determined by sodium dodecyl sulphate (0.1% SDS) polyacrylamide gel electrophoresis and compared with molecular marker (180, 135, 100, 75, 48, 35, 25 kDa)-Sigma.

2.6. Statistical Analysis

Microsoft Excel (Microsoft corporation, USA) was used to analyze data on the average of three replicates (±SE) obtained from three independent experiments, $P < 0.05$.

3. Results

3.1. Dynamics Activity of Endo-1,4-*β*-Glucanase of *Trichoderma harzianum* UzCF 28

Mycelial fungi *T. harzianum* UzCF-28 (**Figure 1**) has a high cellulolytic capacity when grown on medium containing wheat bran as a carbon source. When culturing *T. harzianum* UzCF-28 in the medium of Mendelsa containing 2% wheat bran, for 10 days, there was a significant change in the activity of the cellulase relatively to culturing time. Maximum activity endo-1,4-*β*-glucanase (3.6 units/ml) of fungus detected after 3 days of culturing (**Figure 2**). It is worth to note that growing of the fungus for 6 days resulted in a significant loss of enzyme activity (2.5 units/ml), but on the 7^{th} day of fungus cultivation the enzyme activity increases to 2.8 units/ml. In connection with the complicated regulation of cellulase synthesis of the fungus, it was interesting for us to purify enzymes to homogeneity and study some of their physical and chemical properties.

3.2. Isolation and Purification of the Endo-1,4-*β*-Glucanases of *T. harzianum* UzCF-28

The culture liquid of the fungus *T. harzianum* UzCF-28 was filtered and centrifuged at 3000 g for 30 min. Obtained cell free supernatant served as crude complex of enzymes for further isolation of endo-1,4-*β*-glucanase (EG). Precipitation of the crude enzymes complex was conducted by the method of salting-out using $(NH_4)_2SO_4$ up to 70% saturation and centrifugation at 4000 g for 30 min. Then the precipitate of enzyme complex was dissolved in 50 mM Tris-HCl buffer at pH 6.8, and dialyzed during 24 hours against 100 volumes of 1 mM Tris-HCl buffer at pH 6.8 and 4˚C. It should be noted, that upon each stage of purification of the enzymes extracts were electrophoresed on a polyacrylamide gel. Electrophoresis of the culture liquid and its fractioning by ammonium sulfate have shown 6 and 4 protein stripes, accordingly (**Scheme 1**).

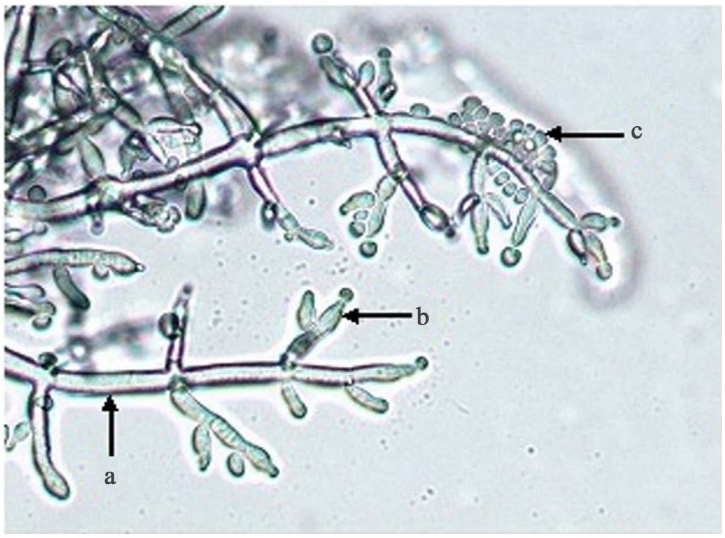


Figure 1. Light microscopy *of T. harzianum* UzCF-28: a—conidiophore, b—phialides, c—conidia.

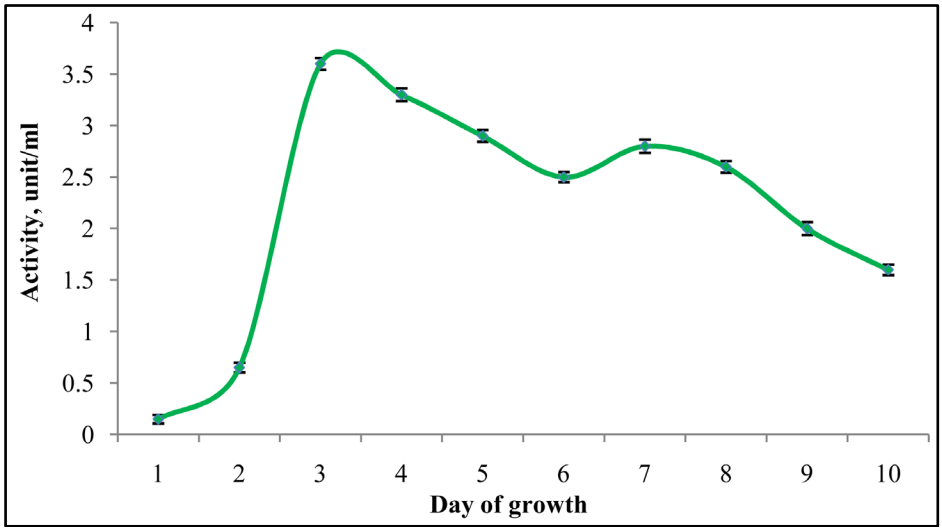
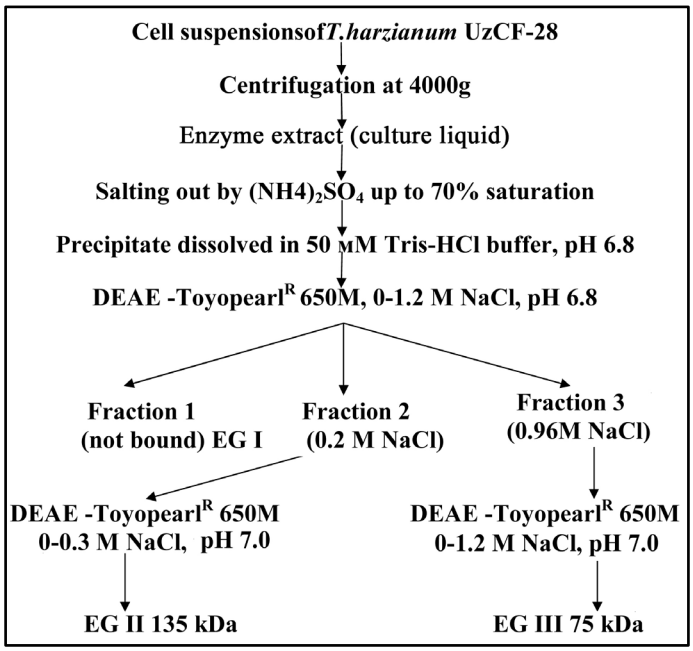


Figure 2. Endo-1,4-*β*-glucanase activity of *T. harzianum* UzCF-28 depending on cultivation time.



Scheme 1. Purification of endo-1,4-*β*-glucanases (EG) of *T. harzianum* UzCF-28.

The next step of purification was ion-exchange chromatography (DEAE-Toyopearl^R 650 M) of dialyzed enzyme preparation. For this purpose 2 × 10 cm-sized column was pre-equilibrated with 50 mM Tris-HCl buffer, pH 6.8. Column was eluated with a partially purified enzyme extract and then the ion exchanger was washed with initial buffer to remove unbound proteins. It should be noted that unbound protein fractions showed endo-1,4-*β*-glucanase (EG I) activity. Similar results were obtained during purification of cellobiohydrolases synthesized by fungus *Chrysosporium lucknowense* [20]. Linear gradient elution of proteins was performed by 50 Tris-HCl buffer with increasing ionic strength (0 - 1.2 M NaCl) at pH 6.8, 40 ml/h of flow rate; the volume of each fraction-2 ml. At this stage of purification two protein peaks with endo-1,4-*β*-glucanase activity were recorded. Each peak contained only one form of the enzyme (**Figure 3**). Activity of EG II was twice higher than EG III's activity. EG II was eluted by 0.2 M NaCl, EG III-by 0.96 M NaCl. During chromatography of proteins it was determined that due to low electric charge of the enzymes EG I they did not bind with ion-exchanger. However, due to high electric charge of molecules EG III bound with gel as strong, so that EG III was eluted

only by high concentration of sodium chloride (0.96 M NaCl),which is nottypical for manyenzymes. During enzyme purification it was revealed that chromatography allows to separatepossibleendo-1,4-*β*-glucanase prote in from the accompanying impurities at pH 6.8, therefore re-chromatography of fractions containing EG II and EG III was carried out on DEAE-Toyopearl^{R} 650 M, at pH 7.0 (**Scheme 1**). As a result of ion-exchange chromatography homogeneous EG II and EG III enzymes were obtained, as confirmed by polyacrylamide gel electrophoresis (**Figure 4(d)**, **Figure 5(a)**). It should be noted that the cleaning of *Thermoascus aurantiacus* endoglucanase was carried out by ion-exchange chromatography on DEAE cellulose DE-52 and gel filtration on Bio-Gel P-100 [7].

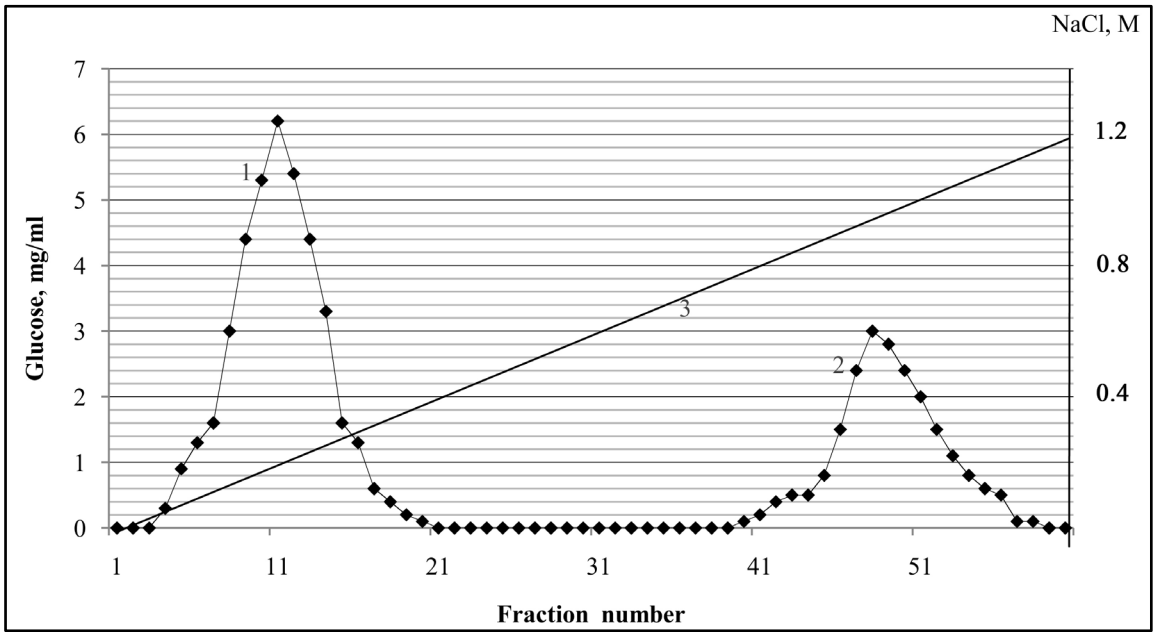


Figure 3. Chromatography of endo-1,4-*β*-glucanases of *T. harzianum* UzCF-28 on DEAE-Toyopearl^{R} 650 M at pH 7.0: 1—EG II, 2—EG III, 3—gradient NaCl.

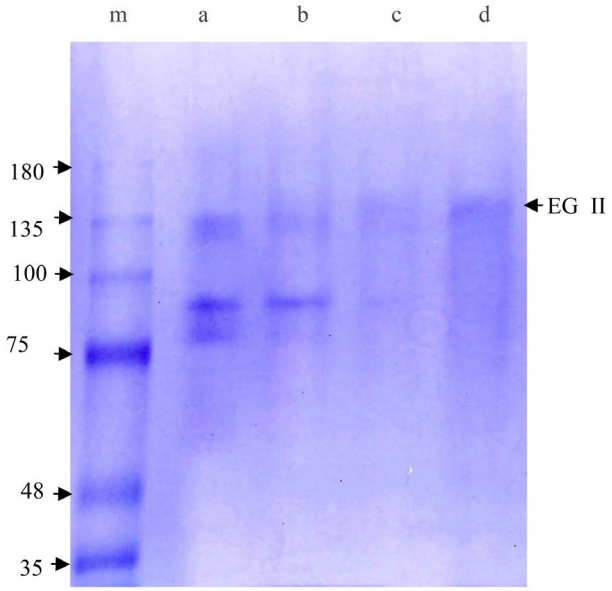


Figure 4. SDS-PAGE proteins after separation on DEAE-Toyopearl^{R} 650 M: m—maker (in kDa), a—fraction 10, b—fraction 11, c—fraction 12, d—purified EG II 135 kDa.

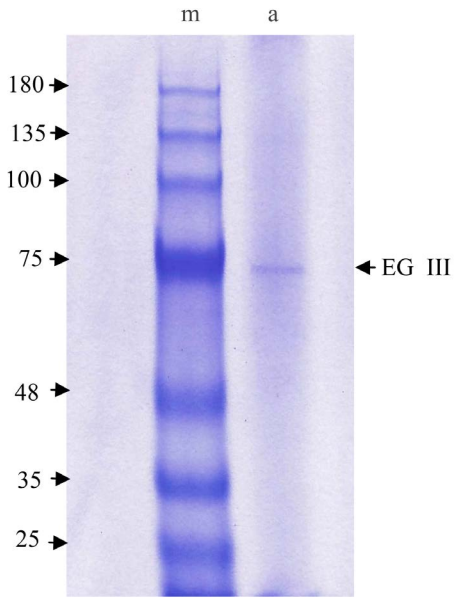


Figure 5. SDS-PAGE: m—maker (in kDa), a—purified EG III 75 kDa.

Molecular weight of endo-1,4-*β*-glucanase isoform determined by electrophoresis on 10% polyacrylamide gel in the presence of 0.1% SDS, indicates that molecular weights of EG II and EG III are nearly equal to 135 and 75 kDa, respectively (**Figure 4(d)**, **Figure 5(a)**). *Trichoderma harzianum* IMI206040 produces at least seven extracellular *β*-1,3-glucanases upon induction with laminarin, a soluble *β*-1,3-glucan. The molecular weights of five of these enzymes were in the range from 60 to 80 kDa [21].

3.3. Physicochemical Properties and Kinetic Characteristics of the Molecular Forms of Endo-1,4-*β*-Glucanase Produced by *T. harzianum* UzCF-28

At present time studies of physiological function of multiplicity of molecular forms of cellulolytic enzymes (endo-1,4-*β*-glucanase, exo-1,4-*β*-glucanase, *β*-glucosidase) of fungi attracts attention of many scientists. One approach to the further research of the issue is a comparative study of physicochemical and kinetic features of the cellulases produced by micromycetes. It may provide some information on physiological role of cellulases in the growth and development of fungi.

In this regard in order to study properties of molecular forms of endo-1,4-*β*-glucanase, enzymes isolated in a homogeneous state from the culture liquid of *T. harzianum* UzCF-28.

During investigation of influence of hydrogen ions concentration on the activity of endo-1,4-*β*-glucanases in a pH range from 3.0 to 8.0 (acetate buffer) it was found that the maximum enzyme activity appears in an acidic medium (**Figure 6** and **Figure 7**). The pH optimum for EG I and EG III was 4.5, while for EG II-4.7, irrespective of the applied substrates—CMC (**Figure 6**) or Whatman filter paper (**Figure 7**). Taha *et al*. [22] during their studies determined that purified cellulolytic enzymes isolated from *T. viride* were active in a wide pH range (4 - 9) and identified that the maximum activity of enzymes was observed at optimum pH value of 6.0. While examinations of Yasmin *et al*. [23] revealed that optimum pH value of purified cellulolytic enzymes isolated from *T. viride* was 4.0.

Investigations of temperature influence on the endo-1,4-*β*-glucanase enzyme activity showed that the optimum temperature of the both enzymes was 50˚C (**Figure 8**). This was also observed on the endo-1,4-*β*-glucanase of *T. viride*, which the optimum temperature is 50˚C [22]. Pandey *et al*. reported the same optimum temperature of 50˚C of cellulolytic enzymes of *T. viride* [11]. It should be noted that the enzyme activity in presence of CMC as a substrate was in two times higher than in presence of Whatmann filter paper (**Figure 8(a)**).

Thermostability of EG I and EG II and EG III was defined by degree of inactivation at 20˚C, 30˚C, 40˚C, 50˚C, 60˚C, 70, 80˚C. For this purpose, 1 ml of enzyme preparation was incubated for 10 minutes at predetermined temperature and then cooled to 5˚C, and enzyme activity was determined at 50˚C for 30 minutes.

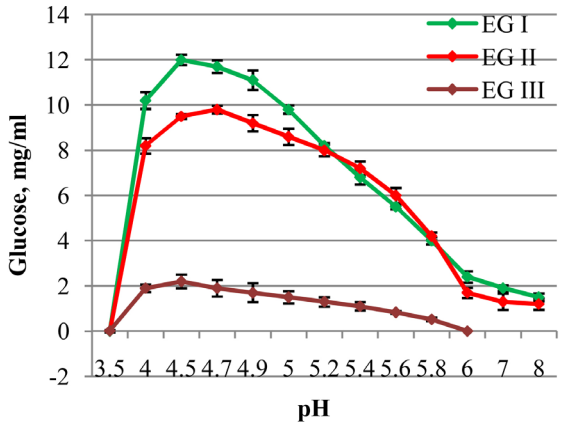


Figure 6. Optimum pH EG I, EG II and EG III in the presence of carboxymethyl cellulose as a substrate.

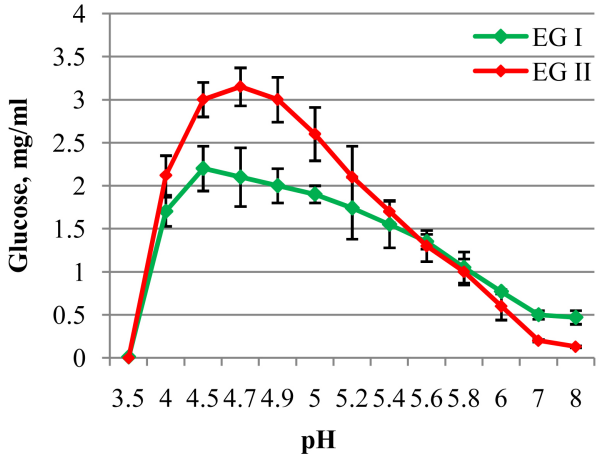


Figure 7. Optimum pH EG I and EG II in the presence of Whatman filter paper as a substrate.

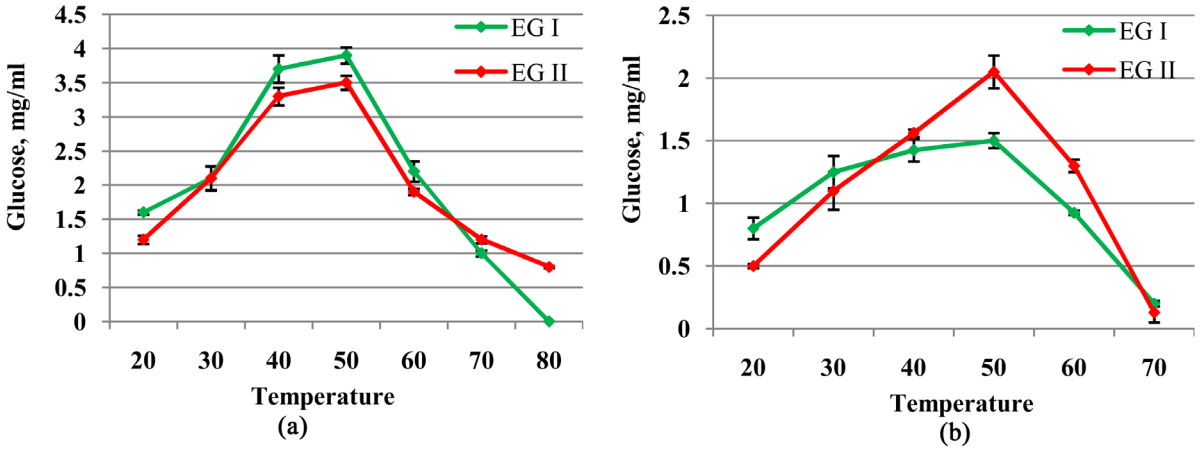


Figure 8. Temperature optimum EG I and EG II in the presence of carboxymethyl cellulose (a) and Whatman filter paper (b) as a substrate.

Experiments have shown that EG I and EG II by heating up to 40˚C were losing activity by 14% and 39%, respectively. Similar results were obtained by Begum M. F. and Absar N. who described that decrease in cellulolytic activity of enzymes at higher temperature might be associated with destruction of enzyme's secondary and

tertiary structures [24]. At the same time, heating up to 40˚C of EG III did not lead to its inactivation, and on the contrary, its activity increased by more than three times comparing to the initial activity of the enzyme, *i.e.* thermo-stability of EG III among tested enzymes significantly varied (**Figure 9(a)**). Similar results were obtained using Whatman filter paper as a substrate (**Figure 9(b)**).

The study of enzyme activity depending on incubation time showed that with prolonging of incubation time up to 60 minutes, the activity of EG I and EG II appropriately increased, irrespective of applied substrates (CMC, Whatman filter paper). Therefore in further experiments determination of endo-1,4-*β*-glucanase activity was performed with incubating the enzymes in reaction mixture for 60 minutes (**Figure 10**). Similar correlation was noted by Chinedu *et al.* [25] who observed hyperbolic increase of activity of purified 1,4-*β*-endoglucanase of *Aspergillus niger* during incubation up to 60 minutes with an interval of 10 minutes.

To determine kinetic characteristics of multiple molecular forms of endo-1,4-*β*-glucanase produced by *T. harzianum* UzCF-28, homogeneous enzymes were also used in the experiments. As it is seen from **Figure 11**, the curve dependant on concentration of CMC is close to hyperbolic. Michaelis K_m constants toward CMC for EG I and EG II are equal to 2 mg and 2.5 mg, respectively (**Figure 11(a)**). The values of K_m towards "Whatman filter" paper for the EG I and EG II are equal to 38 mg and 31 mg (**Figure 11(b)**). Taha *et al.* [22] also describe that increase of substrate concentration leads to increase of enzymatic activity.

Comparing constants of both enzymes it was found that both of them demonstrate high affinity to CMC, than

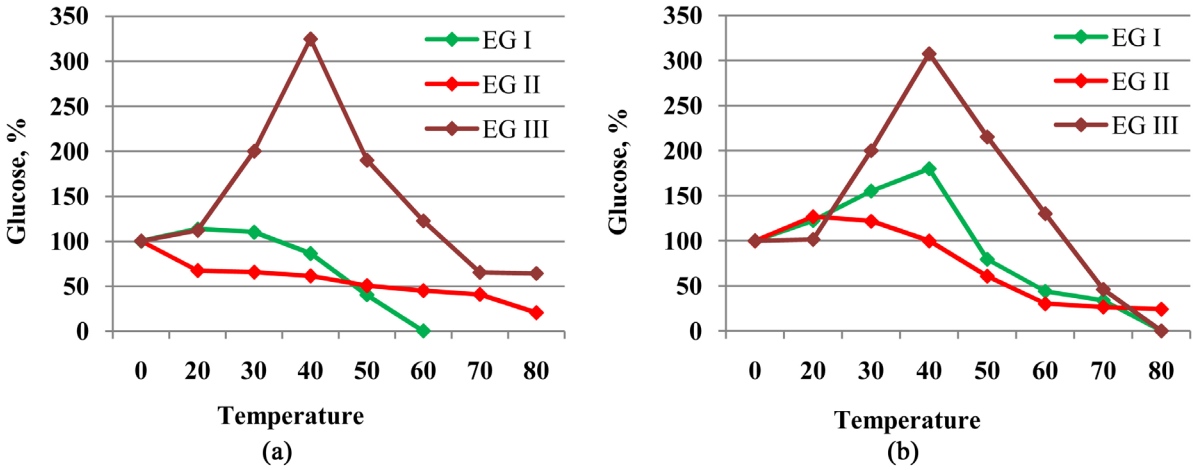


Figure 9. Inactivation of the EG I, EG II and EG III at various temperatures in the presence of carboxymethyl cellulose (a) and Whatman filter paper (b) as substrate.

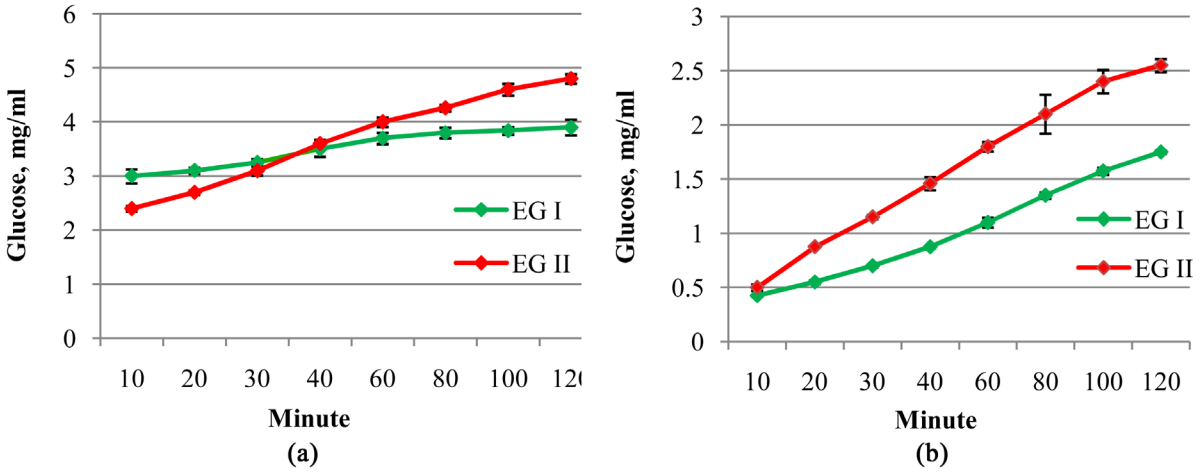


Figure 10. Kinetics of reaction of EG I and EG II at 50˚C in the presence of carboxymethyl cellulose (a) and "Whatman filter" paper (b) as a substrate.

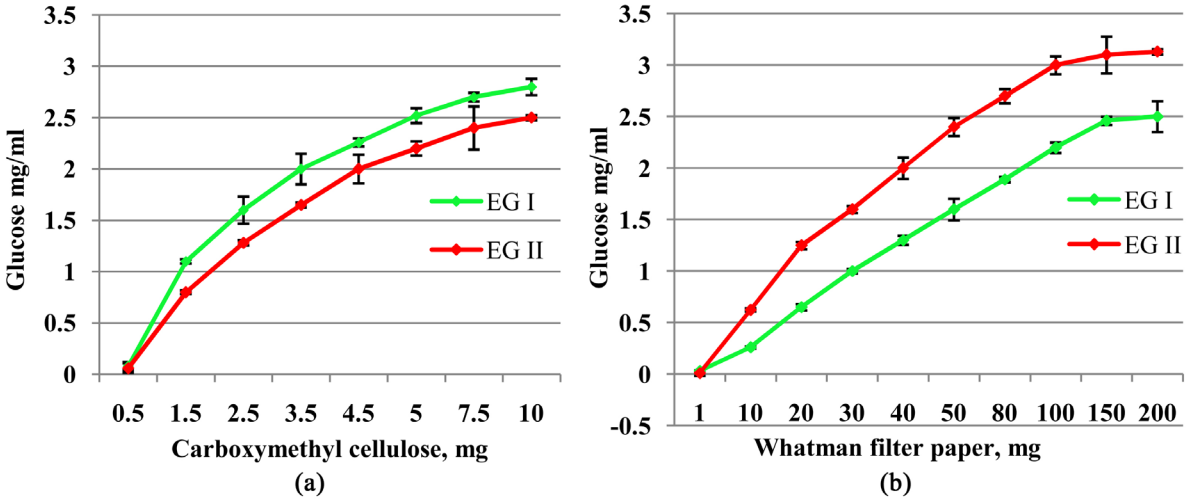


Figure 11. Dependence of an activity of EG I and EG II concentration of carboxymethyl cellulose (a) and "Whatman filter" paper (b) as substrate.

to "Whatman filter" paper. Based on the obtained data, it might be concluded that the investigated enzymes are characterized by significantly lower K_m values towards CMC, than the natural substrates (Whatman filter paper). K_m represents the substrate concentration at which the reaction rate is half of maximal. If the enzyme has a high K_m value, it means that it will take much of the substrate in order to increase the rate of the enzyme catalyzed reaction to half of maximum; low K_m value indicates that the enzyme is saturated with quite small amount of substrate. It also follows that the enzyme-substrate pair with low K_m values are characterized by high affinity for each other and vice versa. In addition, the K_m value helps to found out which substrate groups are most effective in binding to this enzyme.

4. Conclusion

Thus, the obtained data showed that molecular weight of homogeneous endo-1,4-*β*-glucanase 2 (EG II) and endo-1,4-*β*-glucanase 3 (EGIII) of *T. harzianum* UzCF-28 were 135 and 75 kDa, respectively. The isoforms of endo-1,4-*β*-glucanase differ from each other not only in molecular weight and activity levels, but also in many physical and chemical properties and kinetic characteristics. These differences are probably due to differences in the structure of these enzymes synthesized in different cell compartments. Properties like stability at higher temperature for longer period of time and over a wide range of pH showed that the enzymes of *T. harzianum* UzCF-28 can further be used as a potential candidate in various industries.

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