Asian Journal of Chemistry; Vol. 23, No. 3 (2011), 1107-1111

ASIAN JOURNAL OF CHEMISTRY

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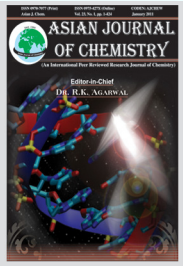

β-Glucosidase from *Lepista flaccida* an Edible Mushroom

H. ELVAN[1], N. SAGLAM ERTUNGA[2,*], A. COLAK[2] and M. YILDIRIM[2]

[1]Resadiye Vocational High School, Gaziosmanpasa University, 60700 Tokat, Turkey
[2]Department of Chemistry, Karadeniz Technical University, 61080 Trabzon, Turkey

*Corresponding author: Fax: +90 462 3253196; Tel: +90 462 3774274; E-mail: nagihanca@yahoo.com

(*Received*: 2 April 2010; *Accepted*: 4 November 2010) AJC-9246

A β-glucosidase was purified 5.0-fold with ammonium sulfate precipitation from the fruiting body of *Lepista flaccida*, an edible mushroom. The enzyme exhibited maximum activity at pH 3.0 and 60 ºC when *p*-nitrophenyl-β-D-glucoside was used as a substrate. K_m and V_{max} values were calculated as 1.06 mM and 5.8 U/mg protein, respectively. The enzyme was quite stable over a broad range of pH (2.0-9.0) at 4 ºC after 24 h incubation. The enzyme activity was conserved about 45 % after 12 h incubation at 60 ºC. Metal ions such as Na^+, Li^+, Mn^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+} and Cu^{2+} and chemicals such as EDTA, phenylmethyl sulfonyl fluoride, β-mercaptoethanol and dithiothreitol had a little negative or positive effect on the enzyme activity. The resistance of the partially purified enzyme to some metal ions and chemicals, along with the pH and thermal stability, can make it very attractive for future applications in industry.

Key Words: Characterization, β-Glucosidase, *Lepista flaccida*, Mushroom.

INTRODUCTION

Cellulose is an unbranched glucose polymer composed of D-glucose units linked by a 1,4-β-D-glucosidic bond, is the most abundant renewable resource on the earth. Renewable alternative energy has recently attracted much attention because of the shortage of fossil fuels, emission of greenhouse gasses and air pollution caused by incomplete combustion of fossil fuels[1]. In addition, the most important process in the production of bioethanol, an important alternative energy resource, is the hydrolysis of cellulose to glucose[2].

Cellulases have attracted much interest because of their enormous potential to convert cellulose to products such as soluble oligosaccharides, glucose, alcohols and other industrially important chemicals[3-5]. The complete enzymatic hydrolysis of cellulosic material needs different types of cellulases *i.e.*, endoglucanase (1,4-β-D-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.74) and β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21)[6]. The endoglucanase randomly hydrolyzes the β-1,4 bonds in the cellulose molecule and the exocellobiohydrolase in most cases releases a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by β-glucosidase[3].

β-Glucosidases are also involved in transglycosylation reactions of β-glucosidic linkages of glucose conjugates. Variations of β-glucosidases are distributed widely in all types of living organisms and play important roles in diverse biological processes depending on the location and the biological system where these reactions occur[7].

The main interest in this enzyme is related to its potential applications in food processing industry (for example, the production of wines and fruit juices) for improving organoleptic product properties[8]. The β-glucosidase is also used in the synthesis of glycoconjugates by reversing the normal hydrolytic reaction[9,10].

The aims of this study are to extract and partially purify a β-glucosidase from the fruiting body of *Lepista flaccida*, an edible mushroom and characterize the enzyme to determine optimum reaction conditions (pH and temperature), thermal and pH stability, kinetic parameters and effects of various chemical compounds. In this way, a new enzyme having industrial applications could be obtained.

EXPERIMENTAL

Lepista flaccida (Sowerby:Fr.) Pat. was collected from Hidirnebi High Plateau (Trabzon, Turkey), carried into the laboratory in liquid nitrogen and stored in deep freeze at -34 ºC. All chemicals used in the study were reagent grade and purchased from Sigma (St. Louis, MO, USA).

Preparation of crude enzyme extract: Crude enzyme extract was prepared as reported previously with some modifications[11-13]. Mushrooms (*ca.* 50 g) were placed in a dewar flask under liquid nitrogen for 10 min in order to decompose cell

membranes. The cold mushrooms were homogenized in 50 mL of 50 mM cold phosphate buffer (pH 7.0) containing 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF) by using a porcelain mortar. After the homogenate was filtered through four layers of muslin, the filtrate was centrifuged at 20,000 rpm for 0.5 h at 4 ºC. The supernatant was used as crude enzyme extract.

Partial purification of *L. flaccida* β-glucosidase: Solid ammonium sulfate was added to the crude enzyme extract to attain 10 % saturation. The mixture was stirred for 2 h at 4 ºC and centrifuged at 20,000 rpm for 0.5 h at 4 °C. While the pellet was redissolved in 50 mM phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer. The supernatant was used to obtain 20 % saturation as explained above. In this way, 30-80 % saturations were also obtained.

Determination of protein concentration: Protein concentration was determined according to the Lowry method with bovine serum albumin as a standard[14]. The values were obtained by graphic interpolation on a calibration curve at 650 nm.

Determination of β-glucosidase activity: β-Glucosidase activity was assayed by using *p*-nitro phenyl-β-D-glucoside (*p*NPG) as a substrate. 200 µL of enzyme solution and 200 µL of *p*NPG (4 mM stock) were incubated for 20 min at 37 ºC. The reaction was stopped by adding 1.2 mL of 0.1 M Na_2CO_3. The amount of *p*-nitro phenol (*p*NP) liberated was estimated at 400 nm. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol *p*NP/min in the reaction mixture under the assay conditions[15].

Activity staining: Non-denaturing polyacrylamide gel electrophoresis was performed at 4 ºC by using an 8 % separating gel containing 4-methylumbelliferyl-β-D-glucopyranoside (MUG) in the final concentration of 0.01 % (w/v). After the electrophoresis was run, the gel was washed three times with 100 mM McIlvaine buffer (pH 5.0) and incubated at 37 ºC for 0.5 h in 50 mM McIlvaine buffer (pH 5.0) containing 0.1 % MUG. β-Glucosidase bands were then visualized under UV light[16].

Effect of pH and temperature on *L. flaccida* β-glucosidase activity: The activity of the partially purified *L. flaccida* β-glucosidase as a function of pH was assayed at 37 ºC by using *p*NPG as a substrate and 50 mM buffer systems with overlapping values: Glycine-HCl (pH 2.0-3.0), McIlvaine (pH 3.0-8.0) and *tris*-HCl (pH 8.0-9.0). The activity was expressed as per cent relative activity with respect to maximum activity, which was considered as 100 %[17].

The optimum temperature of the enzyme was determined at optimum pH value by measuring the activity at different temperatures in the range of 10-80 ºC with 10 ºC increments by using *p*NPG as a substrate. The activity was expressed as per cent relative activity in relation to the temperature optimum, which was considered as 100 %[16].

Enzyme kinetics: Enzyme kinetic parameters of *L. flaccida* β-glucosidase were obtained by measuring the rate of *p*NPG hydrolysis at various substrate concentrations in the standard reaction mixture (at 60 ºC in 50 mM McIlvaine buffer, pH 3.0). The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were determined from the Lineweaver-Burk plot using the Microsoft Excel software.

Effect of pH and temperature on the stability of the enzyme: The effect of pH on the enzyme stability was determined by incubating the partially purified enzyme at 4 ºC for 24 h in the buffer solutions of different pH values: glycine-HCl (pH 2.0), McIlvaine (pH 3.0-8.0) and *tris*-HCl (pH 9.0). At the end of the storage period, the β-glucosidase activity was assayed under standard reaction conditions. The percentage residual enzyme activity was calculated by comparison with non-incubated enzyme[16,18].

In order to determine the thermal stability of the *L. flaccida* β-glucosidase, the enzyme solutions in Eppendorf tubes were incubated at temperatures over the range of 10-60 ºC. Aliquots were withdrawn at times of 0.5, 6, 12, 24 and 48 h, rapidly cooled in an ice bath for 5 min and then brought to 25 ºC. At room temperature, the enzyme activity was determined at standard assay conditions. Control with non-incubated enzyme was used to determine the 100 % activity value[17,18].

Effect of metal ions and chemicals on the enzyme activity: The effect of metal ions on the enzyme activity was separately investigated by adding Na^+, Li^+, Mn^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+} and Cu^{2+} directly to the standard reaction mixture in a final concentration of 1 mM. Enzyme activity determined in the absence of metal ion was defined as 100 %[17].

To study the effect of some chemicals on the enzyme activity, EDTA, PMSF, β-mercaptoethanol (β-ME) and dithiothreitol (DTT) were separately added to the standard reaction mixture in the final concentration of 1 and 10 mM. Also, the effect of sodium dodecyl sulfate (SDS) on the β-glucosidase activity was investigated in the final concentration of 1 and 10 % SDS in the reaction mixture. The percentage residual activities were expressed by comparison with standard assay mixture with no chemical added[7].

RESULTS AND DISCUSSION

Partial purification of *L. flaccida* β-glucosidase: In this study, a β-glucosidase from the fruiting body of *L. flaccida* was partially purified with ammonium sulfate precipitation. It was determined that *L. flaccida* β-glucosidase was purified 5.0-fold with 30-40 % ammonium sulfate saturation and this saturation point was used for performing all other studies such as activity staining, protein determination and enzyme characterization.

Activity staining: Non-denaturing polyacrylamide gel electrophoresis was performed at 4 ºC by using an 8 % separating gel containing MUG in the final concentration of 0.01 % (w/v). After the electrophoresis, β-glucosidase bands were visualized under UV light. While the presence of two β-glucosidase bands were observed in the crude enzyme extract, one activity band was seen in the enzyme solution obtained with 30-40 % ammonium sulfate precipitation (Fig. 1). This means that *L. flaccida* β-glucosidase isoenzymes were successfully separated from each other. Two activity bands observed in the crude enzyme extract could be attributed to the presence of isoenzymes. Existing of isoenzymes for β-glucosidases has been reported for different organisms[19,20].

Effect of pH and temperature on *L. flaccida* β-glucosidase activity: The activity of the partially purified *L. flaccida* β-glucosidase was analyzed at 37 ºC between pH values of

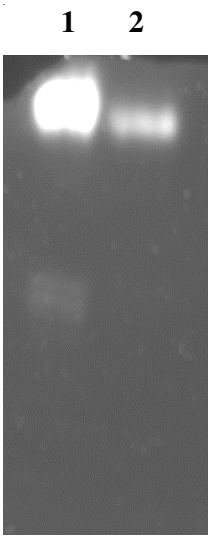

Fig. 1. Non-denaturing polyacrylamide gel electrophoresis

2.0 and 9.0 using 50 mM buffer systems with overlapping values. The optimum pH for the enzyme was 3.0, with 82 and 68 % of the maximum activity appearing at pH 4.0 and 5.0, respectively (Fig. 2).

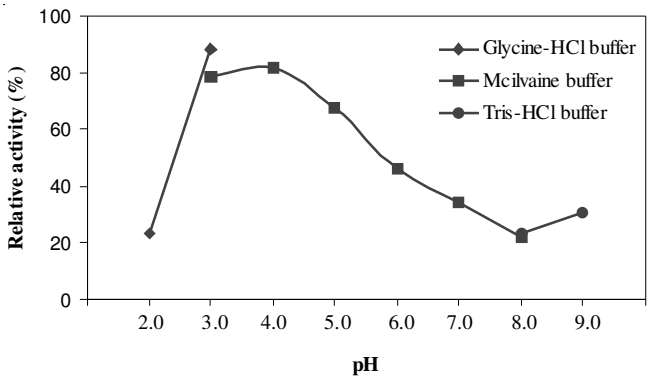

Fig. 2. pH-activity profile of partially purified *L. flaccida* β-glucosidase

Activity of the partially purified enzyme was investigated over a temperature range from 10-80 ºC at pH 3.0. The derived results indicated that the enzyme was active over a broad temperature range with the highest activity at 60 ºC (Fig. 3). Similar results were also reported for *Aspergillus kawachii*, *Stachybotrys*, *Aspergillus oryzae* and *Periconia* sp. β-glucosidases[7,21-23].

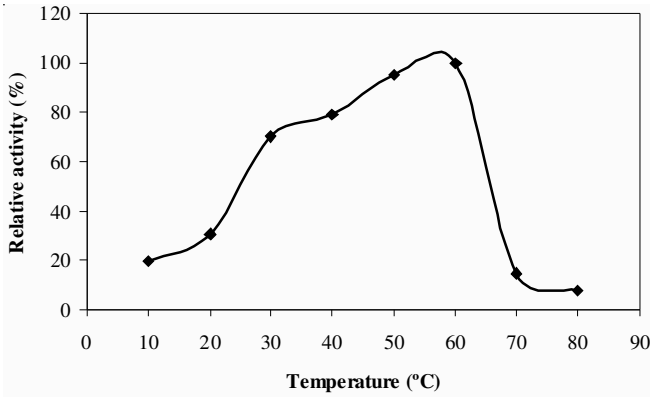

Fig. 3. Temperature-activity profile of partially purified *L. flaccida* β-glucosidase

Enzyme kinetics: The effect of *p*NPG concentration on the *L. flaccida* β-glucosidase activity was measured at substrate concentrations ranging from 0.1-5.0 mM. The values of K_m and V_{max} were calculated from Lineweaver-Burk plot as 1.06 mM and 5.8 U/mg protein, respectively (Fig. 4). The enzyme had a comparable K_m value of 1.06 mM for *p*NPG. In comparison, *p*NPG, K_m values of purified β-glucosidases from other organisms range from 0.20-21.7 mM[24].

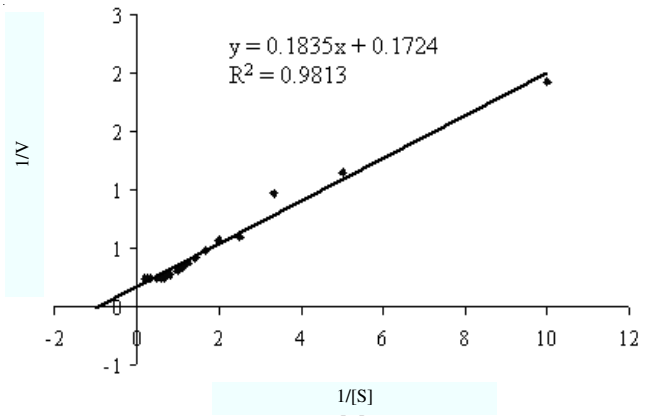

Fig. 4. Lineweaver-Burk plot of *L. flaccida* β-glucosidase. Enzyme kinetic parameters of the enzyme were obtained by measuring the rate of *p*NPG hydrolysis at various substrate concentrations at 60 ºC in 50 mM McIlvaine buffer (pH 3.0). K_m and V_{max} values were determined from the Lineweaver-Burk plot using the Microsoft Excel software

Effect of pH and temperature on the stability of the enzyme: pH stability of *L. flaccida* β-glucosidase was determined by incubating the partially purified enzyme at 4 ºC in the buffers having different pH values for 24 h. After incubation, the activity was assayed under standard reaction conditions. The enzyme retained over 90 % of its original activity at pH 4.0 and 5.0. It was also quite stable at pH 2.0-3.0 and 6.0-9.0 after 24 h incubation (Fig. 5). It was reported that 90 % of *A. oryzae* β-glucosidase activity remained after 17 h incubation at pH 5.0-7.0 at 30 ºC. However, only about 70 % of the activity remained at pH 4.0[25]. More than 70 % of the origi-nal activity of purified *Fusarium proliferatum* ECU2042 remained after 24 h incubation at pH 4.0-6.5 at 4 ºC[26]. *Periconia* sp. β-glucosidase was also stable under basic conditions, retaining almost 100 % of maximal activity after incubation for 2 h at pH ≥ 8[7].

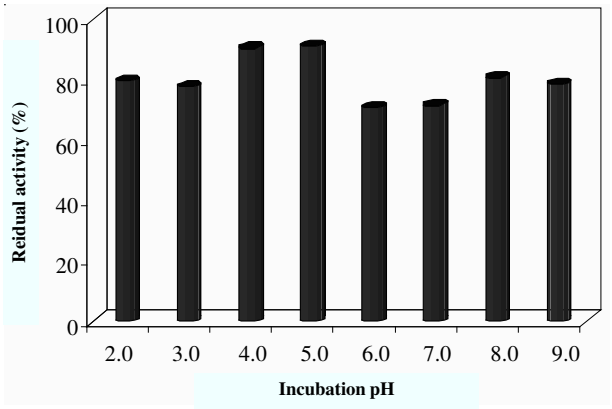

Fig. 5. pH profile of partially purified *L. flaccida* β-glucosidase

Thermal stability profile for the *L. flaccida* β-glucosidase, presented in the form of the residual activity, is shown in Fig. 6(a-b). When the enzyme was incubated at temperatures between 10 and 40 ºC for 0.5 h, it retained almost 80 % of its original activity, but it lost 35 and 37 % of its original activity at 50 and 60 ºC, respectively (Fig. 6a). While the enzyme activity was conserved about 45 % after 12 h incubation at optimum temperature value, the enzyme retained its original activity approximately 70, 55 and 45 % after 48 h incubation at 10, 20 and 30 ºC, respectively (Fig. 6b). It was reported that β-glucosidase from *Volvariella volvacea*, an edible mushroom, lost 70 % of its original activity after only 5 min incubation at 60 ºC[27]. Activity of *A. oryzae* β-glucosidase significantly decreased at temperatures above 40 ºC when the enzyme was incubated for 4 h[25]. A β-glucosidase from *Paecilomyces thermophila* was highly thermostable at 50 ºC and retained more than 95 % of the initial activity after 8 h. At 60 ºC, it retained more than 70 % of the initial activity after 8 h incubation[28]. *Periconia* sp. β-glucosidase was found to retain approximately 60 % of its maximal activity for at least 2 h at 30-60 ºC. After incubation at 70 ºC, it retained more than 50 % activity for 2 h[7]. The residual activity of *Debaryomyces pseudopolymorphus* β-glucosidase after 3 h incubation at 40 ºC decreased to approximately 30 % of the maximum activity[29].

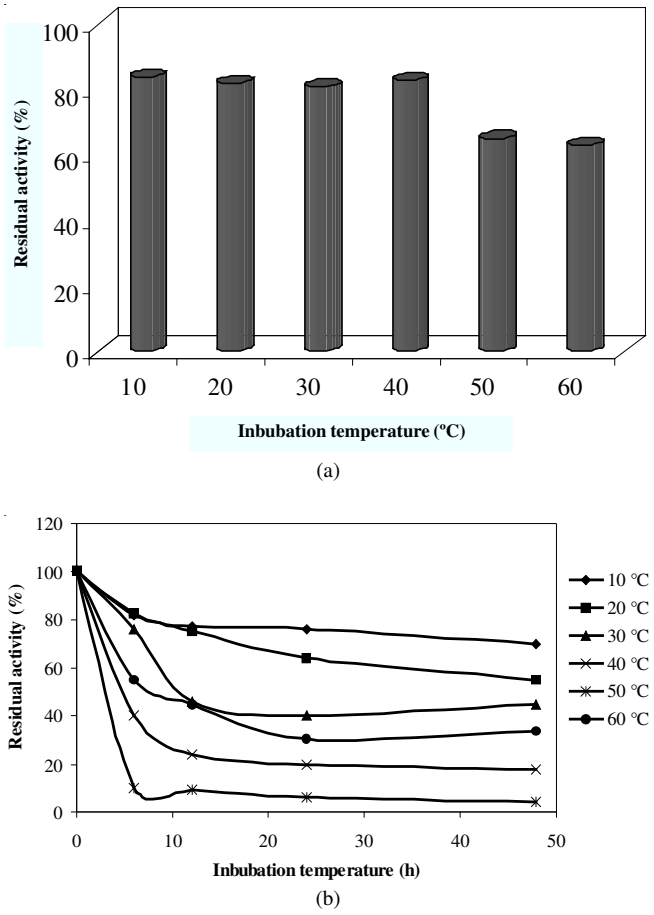

Fig. 6. Thermal stability profile of partially purified *L. flaccida* β-glucosidase

Effect of some metal ions and chemicals on the enzyme activity: The effect of Na^+, Li^+, Mn^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+} and Cu^{2+} on the β-glucosidase activity of *L. flaccida* was tested at 1 mM concentration of each metal ion. All had a little negative or positive effect on the enzyme activity, demonstrating that the enzyme did not require these metal ions to function (Table-1). It was reported that *F. proliferatum* ECU2042 β-glucosidase was slightly inhibited in the presence of 10 mM Zn^{2+} [26]. *Fomitopsis pinicola* β-glucosidase[24] was also inhibited by 0.1 mM Co^{2+}.

TABLE-1
EFFECT OF SOME METAL IONS ON THE β-GLUCOSIDASE ACTIVITY OF *L. flaccida*

Metal ion	Residual activity (%)
None	100
Na^+	98
Li^+	97
Mn^{2+}	103
Zn^{2+}	95
Hg^{2+}	100
Co^{2+}	98
Cu^{2+}	103

To study the effect of EDTA, PMSF, β-ME, DTT and SDS on the enzyme activity, they were separately added to the standard reaction. Except for SDS, all chemicals slightly affected the β-glucosidase activity. The enzyme activity was almost fully inhibited by SDS (Table-2). The little inhibition action of the chelating agent EDTA in the final concentration of 10 mM allowed us to conclude that the active site of this enzyme is not dependent on divalent cations for enzyme activation. The inhibition of the enzyme by SDS indicates that the integrity of its three-dimensional structure is critical for its catalytic activity. It was reported that the activity of *Periconia* sp. β-glucosidase slightly increased by 0.1 % β-ME and severely inhibited by 1 % SDS[7].

TABLE-2
EFFECT OF CHEMICALS ON THE β-GLUCOSIDASE ACTIVITY OF *L. flaccida*

Chemical	Residual activity (%)	
	1 mM final conc.	10 mM final conc.
None	100	100
EDTA	102	98
PMSF	96	95
β-ME	94	92
DTT	98	96
	1 % final conc.	10 % final conc.
SDS	3	1

Conclusion

A pH and thermostable β-glucosidase was partially purified from *L. flaccida* with ammonium sulfate precipitation and characterized. The high thermostability of *L. flaccida* β-glucosidase is advantageous, since reaction at elevated temperature provide an opportunity for increased solubility of reactants and products, resulting in higher enzymatic activity as well as higher reaction velocity stemming from lower viscosity. In addition, there is less energy cost for cooling when thermal pre-treatment of substrates was required or desirable such as steam explosion of biomass in biorefining process. The risk of contamination is also reduced. Because of thermostable cellulytic enzymes

also have great potential to be used in industrial processes such as food processing, textiles and bioconversion[3,30], *L. flaccida* β-glucosidase could be useful for these industrial applications.

ACKNOWLEDGEMENTS

This work was financially supported by KTU-BAP to NSE (2007.111.002.10). The authors thank Prof. Dr. Ertugrul Sesli (Department of Science Education, Karadeniz Technical University, 61335 Trabzon, Turkey) for providing and identifying the mushroom.

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