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PARTIAL PURIFICATION AND CHARACTERIZATION OF β-GLUCOSIDASE FROM *ALOE VERA* L. LEAVES

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SUMMARY

The partial purification of β-glucosidase from the pulp of *Aloe vera* L. Burm. fil. (sarisabir) leaves and some of its kinetic properties is presented. The fresh leaves of *A. vera* were used; the gel portion was separated and the remaining leaf pulps were cut into small pieces. The crude extract was prepared by homogenization of the leaf pulps in phosphate buffered saline (PBS), pH 7.4 and subsequent centrifugation. β-Glucosidase active fraction was precipitated by 30%-65% ammonium sulphate from the crude extract. Hydroxylapatite column chromatography resulted in a single peak showing β-glucosidase activity eluted with 200 mM phosphate buffer. The partially purified enzyme showed two protein and a single activity band in polyacrylamide gel electrophoresis. It was found that the enzyme exhibited maximum activity at 50°C and at pH 4.4. Km and Vmax values for 4-nitrophenyl-β-D-glucopyranoside were $6.8x10^{-4}$ M and $4,58x10^{-3}$ U, respectively. When β-glucosidase activity was investigated throughout the year, it was found that the activity increased in winter and decreased in summer.

ÖZET

Aloe vera L. Burm. fil. (sarısabır) yapraklarının pulpa kısmından β-glukozidaz kısmen saflaştırıldı ve bazı kinetik özellikleri incelendi. *A.vera*'nın taze yaprakları

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kullanıldı; jel kısmı ayrıldı ve kalan yaprak pulpaları küçük parçalara kesildi. Yaprak pulpalarının fosfatla tamponlanmış %0.9 NaCl (PBS), pH 7.4 ile homojenizasyonunun ardından santrifüje edilmesiyle ham ekstre hazırlandı. β-Glukozidaz aktivitesi gösteren fraksiyon ham ekstreden %30-%65 amonyum sülfat ile çöktürüldü. Hidroksilapatit kolon kromatografisi sonucunda 200 mM fosfat tamponu ile β-glukozidaz aktivitesi gösteren tek bir pik elde edildi. Kısmen saflaştırılan enzim poliakrilamid jel elektroforezinde iki protein ve bir aktivite bandı gösterdi. Enzimin en yüksek aktiviteyi 50°C'de ve pH 4.4'te gösterdiği ve 4-nitrofenil-β-D-glukopiranozide karşı Km değerinin $6.8x10^{-4}$ M, Vmax değerinin ise $4.58x10^{-3}$ U olduğu saptandı. β-Glukozidaz aktivitesi yıl boyunca incelendiğinde aktivitenin kış aylarında arttığı, yaz aylarında ise azaldığı bulundu.

Key words: glycosidases, β-glucosidase, *Aloe vera,* purification, seasonal variation

INTRODUCTION

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) occurs widely in prokaryotes and eukaryotes. It catalyzes the hydrolysis of the β-glucosidic linkages of di- and/or oligo-saccharides, or other glucose conjugates. In plants, β-glucosidase activity is implicated in many important biological processes (1). Specific plant β-glucosidases are involved in chemical defense against pathogens and herbivores via the process of cyanogenesis, whereby HCN and other toxic compounds are released upon hydrolysis of cyanogenic glucosides (2). Other plant β-glucosidases are implicated in regulating the biological activity of plant phytohormones such as cytokinin, gibberellin, and auxin by releasing active forms from inactive hormone-glucoside conjugates (3). In fungi and bacteria, β-glucosidase is involved in the hydrolysis of cellobiose into two molecules of glucose. Cellobiose itself is the degradation product of cellulose and its accumulation inhibits the activity of cellulases; therefore, β-glucosidase plays an important role in the saccharification of cellulose by removing cellobiose (1). β-Glucosidases have been purified and characterized from a variety of cereals, such as rice (4), rye (5), wheat (6) and fruits such as orange (7), grape (8), sweet cherry (9), and also different strains of fungi (10,11).

Aloe vera L. Burm. fil. (= *A. barbadensis* Miller) (Aloaceae) is native in North Africa and is also cultivated in Turkey. *Aloe* species have been used for centuries for their laxative, antiinflammatory, immunostimulant, antiseptic (12), wound and burn healing (13), antiulcer (14,15), antitumour (15,16) and antidiabetic (17,18) activities. To our knowledge, this is the first study dealing with the separation and characterization of β-glucosidase from *Aloe vera* leaves.

RESULT AND DISCUSSION

Between the glycosidases investigated in *A. vera* leaf pulp extract (α-, β-glucosidase and α-, β-galactosidase), β-glucosidase was shown to possess the highest activity (Table 1). On the other hand the activity of the same enzymes in *A. vera* leaf gel extract was very low (data not shown). It was accordingly decided to purify β-glucosidase from *Aloe vera* leaf pulp. Esen (1), indicated that β-glucosidases played pivotal roles in many metabolic processes of plant such as growth, productivity and defence.

Table 1. Total activity values of *Aloe vera* leaf pulp enzymes.
(The values were obtained from one *A. vera* leaf, 415 g)

Enzyme	Total Activity (U)*
β-Glucosidase	60873
α-Glucosidase	23673
α-Galactosidase	10728
β-Galactosidase	27362

(*) μmol of 4-nitrophenol/min

Elution of ammonium sulphate fraction with 200 mM phosphate buffer (pH 7.0) through hydroxylapatite column resulted in one β-glucosidase active peak (Fig. 1).

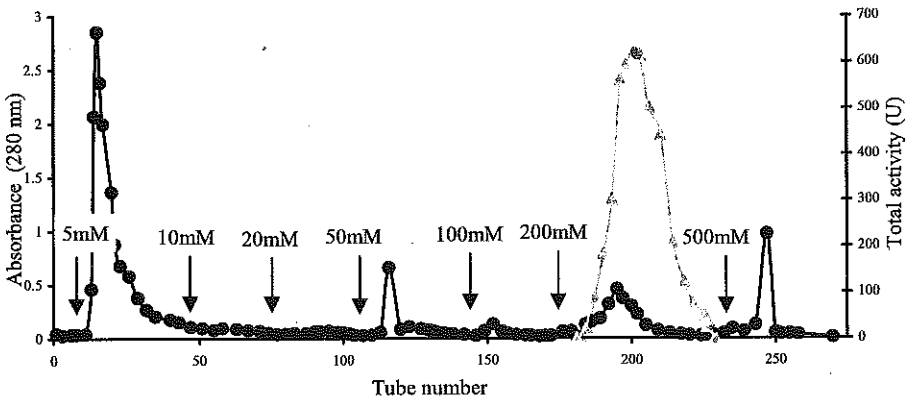

Figure 1. Hydroxylapatite column chromatography elution profile of the fraction obtained by 30%-65% ammonium sulphate saturation of the crude leaf pulp extract. Column dimensions: 2x15 cm, applicate: 36 mg protein, flow rate: 48 mL/hr.
Protein at 280 nm (—●—), activity of β-glucosidase ( )

Protein contents and β-glucosidase activities of the samples were determined at each purification step and the results are presented in Table 2. The purified enzyme was enriched 28-fold (Table 2).

Table 2. Partial purification of *Aloe vera* leaf pulp β-glucosidase. (Starting with 448 g leaf pulp obtained from 1653 g fresh leaves)

Purification step	Total protein (mg)	Total activity (U)*	Specific activity (U/mg)**	Purification factor
Crude extract	2402	69988	29	1
30%-65% $(NH_4)_2SO_4$ cut	203	91920	453	16
Hydroxylapatite (200mM buffer eluate)	15.4	12401	805	28

(*) μmol of p-nitrophenol/min
(**) μmol of p-nitrophenol/min/mg protein

In polyacrylamide gel electrophoresis, the purified β-glucosidase gave two protein bands (Amido Black 10B staining) and a single activity band (4-nitrophenyl-β-D-glucopyranoside staining) at the same migration distance with the protein band at the top (Fig. 2). The occurance of a single activity band suggests that the purified β-glucosidase do not contain isoenzymes just as the β-glucosidase purified from orange (7) and sweet cherry (9). In contrast, it was reported that several β-glucosidase isoenzymes were obtained from rye (5) and wheat (6).

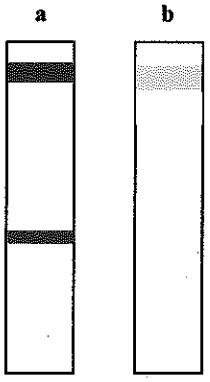

Figure 2. The schematic presentation of polyacrylamide gel electrophoresis of the purified β-glucosidase. Gel **a** stained for protein, gel **b** stained for activity.

When the effect of temperature on the activity of fungal (10,11,19) and plant (4,7,8,20,21) β-glucosidases were researched, it was found that β-glucosidase exhibited

the highest activity at 60-70°C and 40-50°C respectively. As can be seen, when thermal stability of β-glucosidases of both sources were compared, fungal β-glucosidases were much more resistant to heat than plant glucosidases. In present study we found that *Aloe vera* leaf pulp β-glucosidase showed a maximal activity at 50°C, similar to values of other plant β-glucosidases (Fig. 3).

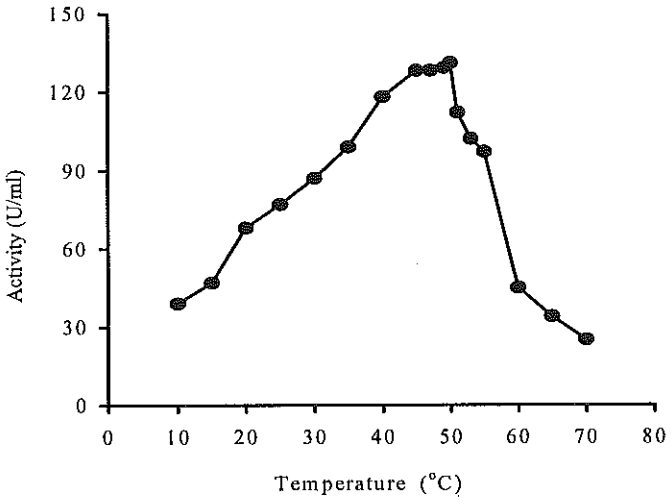

Figure 3. Effect of temperature on the β-glucosidase activity.

The purified β-glucosidases may have different optimum pH values according to their sources. In this study β-glucosidase showed the highest activity at pH 4.4 (Fig. 4). This optimum pH value is similar to values of rice (pH 4.5) (4) and orange (pH 4.5-5.5) (7) β-glucosidases. Also fungal β-glucosidases (10,11,19) exhibited a maximal activity at asidic pH (4.5-5.5) like plant β-glucosidases. In contrast to plant β-glucosidases that showed optimum activity at asidic pH, β-glucosidase purified from vanilla bean exhibited a maximal activity at pH 6.5 (20).

The Km and Vmax values of β-glucosidase for 4-nitrophenyl-β-D-glucopyranoside were 6.8×10^{-4} M, 4.58×10^{-3} U, respectively. These results indicated that β-glucosidase had high affinity for this substrate. When the substrate specificity of other plant β-glucosidases was investigated, these enzymes showed high affinity not only for their natural substrates but also for aryl glucosides such as 4-nitrophenyl-β-D-glucopyranoside (22,23).

Little information on seasonal variation for plant glucosidase activity have been found in literature. Sivakumar *et al.*, have reported maximum β-glucosidase activity in uninfected olive fruits during October (24). In this study, β-glucosidase activity had

been investigated throughout the year and maximum activity was observed in November and the activity decreased in the summer months (Table 3). The raise of activity in winter may be due to the need for β-glucosidase because of environmental stress such as cold and food poverty.

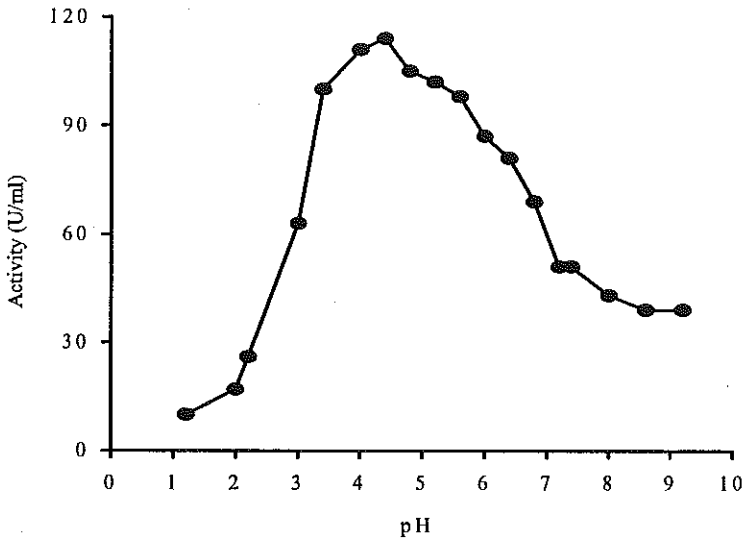

Figure 4. Effect of pH on the β-glucosidase activity.

We conclude that *Aloe vera* leaf pulp can be an important source for β-glucosidase and the most suitable time for this purification is in winter months. The aim for the future is further purification and characterization of *Aloe vera* leaf pulp β-glucosidase in order to understand the events occurring during the plant's metabolism.

Table 3. β-Glucosidase activity of *Aloe vera* leaf pulp crude extract throughout the year.

Months	Activity (U/mL)	Specific activity (U/mg)
May	129	23
July	96	30
September	86	31
November	762	231
January	320	228
March	415	92

EXPERIMENTAL

Plant material: Specimens of *Aloe vera* (L.) Burm. fil. (in Turkish 'Sarisabir') were collected from Kale (Demre) in Antalya, identified by Prof.Dr. N. Sütlüpinar and cultivated in the greenhouse of the Botanical Garden of Istanbul University, Faculty of Sciences. In this study the fresh leaves of this cultivated plant were used. A voucher specimen of the plant was deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE). Herbarium code number: ISTE-65118.

Chemicals: Hydroxylapatite was prepared in our laboratory according to Tiselius *et al.* (25). All other chemicals used were analytical reagent grade.

Determination of occurence of glycosidase activities in *Aloe vera* leaf pulp and gel:

1-Preparation of the extracts. One *Aloe vera* leaf (415 g) was washed and cut open from the middle, the gel was separated by scraping with a spoon and homogenized in a Waring commercial blender then filtered through cloth **(leaf gel extract)**. The remaining pulps (103 g) were cut into small pieces and homogenized in a Waring commercial blender with PBS. The homogenate was filtered through cloth and the filtrate was centrifuged at 6000 rpm for 30 min at 4°C in a refrigerated centrifuge (Megafuge 1.0R Heraeus). The green pellet was discarded and the clear yellow supernatant **(crude leaf pulp extract)** was selected.

2-Enzyme assay. α-Glucosidase, β-glucosidase, α-galactosidase and β-galactosidase activities were determined by incubating 0.1 mL of 20 mM p-nitrophenyl-α-glucopyranoside, p-nitrophenyl-β-glucopyranoside, p-nitrophenyl-α-galactopyranoside, p-nitrophenyl-β-galactopyranoside, respectively with 0.1 mL of Mc Ilvaine citrate-phosphate buffer (pH 4.8) and 0.1 mL of enzyme solution for 30 min at 37°C. The reaction was stopped by the addition of 3 mL of 0.2 M Na_2CO_3 and the colour developed by p-nitrophenol liberation was measured at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol from the substrate per minute under standart assay conditions.

Purification of β-glucosidase:

1-Preparation of the crude extract. *Aloe vera* L. leaves (1653 g) were washed and cut open from the middle, the gel was separated by scraping with a spoon. The crude extract was prepared with the remaining pulps (448 g) as describe above.

2- Ammonium sulphate fractionation. The crude extract was saturated to 30% with ammonium sulphate crystals and left overnight at 4°C. The precipitate formed was separated by means of centrifugation at 6000 rpm (4°C) for 30 min and discarded. The supernatant (initial 30%) was saturated this time to 65% by adding ammonium sulphate

crystals. The precipitate (65% saturation), thus obtained was left overnight in 4°C separated by centrifugation at 6000 rpm (4°C) for 30 min, dissolved in PBS by magnetic stirring and dialysed against the 1 mM phosphate buffer **(30%-65% $(NH_4)_2SO_4$ cut)**.

3-Hydroxylapatite column chromatography. 16 mL dialysate (30%-65% $(NH_4)_2SO_4$ cut, 36 mg protein) was applied to a hydroxylapatite column (2x15 cm). The elution was performed by washing the column stepwise with 5 mM, 10 mM, 20 mM, 50 mM, 100 mM, 200 mM and 500 mM Na-K phosphate buffer (pH 7.0), each buffer volume was three times the column volume. The fractions were analyzed for protein and β-glucosidase activity. Then the fractions exhibiting enzyme activity were pooled separately. The whole procedure was accomplished at 4°C.

Protein determination: Protein contents of the samples obtained during the extraction and precipitation processes were determined by method of Lowry *et al.* (26) using bovine serum albumin as a standard. Proteins in column eluates were measured by the E280/260 method (27).

Polyacrylamide gel electrophoresis (PAGE): PAGE (250 μg for protein staining and 320 μg for activity staining of the purified β-glucosidase) was performed in Acrylophor Pleuger electrophoresis cell according to Ornstein (28) and Davis (29) on 7.5% acrylamide gels. The gels were stained with Amido Black 10B to determine the protein band. The β-glucosidase activity was revealed in native PAGE gels with 4-nitrophenyl-β-D-glucopyranoside (30).

Kinetic Properties of β-glucosidase:

1- Effect of temperature on enzyme activity. The optimum temperature was determined by measuring the β-glucosidase activity under standard assay conditions in the temperature range 10-70°C with a temperature interval of 5°C.

2- Effect of pH on enzyme activity. Variation in the activity of β-glucosidase according to pH changes was determined over the pH range of 1.2-9.2 under standard assay conditions. Sϕrensen glycine, McIlvaine citrate-phosphate and Sϕrensen phosphate buffers were used for 1.2-3.6, 2.2-7.2, 7.0-9.2 pH ranges, respectively with pH intervals of 0.2.

3- Substrate specificity. The dependence of the β-glucosidase activity on the various substrate concentrations was investigated under standard assay conditions by using 20 mM, 10 mM, 5 mM, 2.5 mM and 1.25 mM 4-nitrophenyl-β-D-glucopyranoside. The values of Michaelis constants (Km) and maximum velocity (Vmax) were calculated by means of the equation of Lineweaver and Burk plots.

Seasonal variation of β-glucosidase activity:

Fresh *Aloe vera* leaves were collected from the greenhouse with two months intervals throughout the year and pulp extracts were prepared. β-Glucosidase activity and protein contents of these extracts were measured.

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