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Purification of beta-glucosidase from olive (*Olea europaea* L.) fruit tissue with specifically designed hydrophobic interaction chromatography and characterization of the purified enzyme

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ABSTRACT

An olive (*Olea europaea* L.) β-glucosidase was purified to apparent homogeneity by salting out with ammonium sulfate and using specifically designed sepharose-4B-L-tyrosine-1-napthylamine hydrophobic interaction chromatography. The purification was 155 fold with an overall enzyme yield of 54%. The molecular mass of the protein was estimated as ca. 65 kDa. The purified β-glucosidase was effectively active on *p*-/*o*-nitrophenyl-β-D-glucopyranosides (*p*-/*o*-NPG) with K_m values of 2.22 and 14.11 mM and V_{max} values of 370.4 and 48.5 U/mg, respectively. The enzyme was competitively inhibited by δ-gluconolactone and glucose against *p*-NPG as substrate. The K_i and IC_{50} values of δ-gluconolactone were determined as 0.016 mM and 0.23 mM while the enzyme was more tolerant to glucose inhibition with K_i and IC_{50} values of 6.4 mM and 105.5 mM, respectively, for *p*-NPG. The effect of various metal ions on the purified β-glucosidase was investigated. Of the ions tested, only the Fe^{2+} increased the activity while Cd^{2+} Pb^{2+} Cu^{2+}, Ni^{+}, and Ag^{+} exhibited different levels of inhibitory effects with K_i and IC_{50} values of 4.29×10^{-4} and 0.38×10^{-4}, 1.26×10^{-2} and 5.3×10^{-3}, 2.26×10^{-4} and 6.1×10^{-4}, 1.04×10^{-4} and 0.63×10^{-4}, 3.21×10^{-3} and 3.34×10^{-3} mM, respectively.

1. Introduction

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) selectively catalyzes the hydrolytic cleavage of β-glycosidic linkage between two or more glycone residues or of that between glucose and an aryl or alkyl aglycone. The enzyme constitutes a major group among glycoside hydrolases that occur universally in all living organisms from bacteria to humans. β-Glucosidases play key roles in a variety of essential physiological processes and potential biotechnological applications depending on the nature and diversity of the glycone or aglycone moiety of their substrates. Among the mammalian β-glucosidases, the human acid β-glucosidase, commonly known as glucocerebrosidase, catalyzes the degradation of glucosylceramide in the lysosome. The inherited deficiency of the enzyme leads to Gaucher's disease [1]. β-Glucosidases in cellulolytic microorganisms have recently been the focus of much research since cellulose is the most abundant substrate on earth and is very likely to be an important renewable resource of energy in the future [2–4]. Plant β-glucosidases have been reported to be involved in regulation of the physiological activity of phytohormones by hydrolysis of their inactive hormone–glucoside conjugates [5,6], chemical defense against pests [7–9], lignification [10], β-glucan synthesis during cell wall development and cell wall degradation in the endosperm during germination [11,12], and food quality and flavor enhancement [13].

Due to nutritional and health benefits, olives and their oil have been in high demand especially in the last decade. It was known that phenolic compounds such as oleuropein, demethyloleuropein, verbascoside and luteolin-7-glucoside found in olives, have an effective health role [14–16]. β-Glucosidases catalyze the hydrolysis of these phenolic glucosides. The present paper mainly describes a newly developed purification strategy for the purification of the enzyme protein and of its characterization as a β-glucosidase in olive fruit.

2. Materials and methods

2.1. Plant material

Olive fruits (*Olea europaea* L.) used for this research were collected, 32 weeks after flowering, in Seferihisar-Izmir.

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2.2. Chemicals

Sepharose-4B, L-tyrosine, 1-napthylamine, *p*-NPG, *o*-NPG, protein assay reagents and chemicals for electrophoresis were purchased from Sigma–Aldrich (Germany). Inorganic salts were supplied by Merck. All other chemicals were of the best available grade.

2.3. Extraction of the enzyme

After thoroughly washing the freshly harvested olives, the seeds were removed and acetone powders were prepared from the pulp tissue. Pulp tissue (100 g) was ground for 2 min in 1500 mL of cold acetone (−20 °C) using a Waring blender [16]. The residue obtained after filtration, through Whatmann 1 MM filter paper, was re-extracted three times with 500 mL of cold acetone (−20 °C). The last residue on the filter paper was transferred to petri dishes and air dried at room temperature for about 5 h. The reddish-purple powder obtained was stored at −20 °C for further use.

Enzyme extracts were prepared from 0.5 g of the above powder in 50 mL of cold buffer (4 °C) containing 100 mM borate buffer (pH 9.0), 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.25% (w/v) dithiothreitol (DTT) using a Miccra XRT homogenizer [16]. The homogenate was centrifuged at 15,300 rpm for 30 min at 4 °C, and the supernatant was used as the crude extract.

2.4. Purification of the enzyme

All purification steps were performed at 4 °C unless otherwise stated. The crude enzyme was treated with solid ammonium sulfate to obtain the 0–50% saturation fraction by centrifuging at 15,000 rpm for 30 min. The precipitate was dissolved in 50 mM sodium phosphate buffer (pH 6.8) and the final saline concentration was adjusted to 1 M ammonium sulfate prior to applying to the sepharose-4B-L-tyrosine-1-napthylamine column.

The hydrophobic column was prepared using 10% CNBr in a 1:1 suspension of sepharose 4B and water. The mixture was adjusted to pH 11 in an ice bath and maintained at that pH for 8–10 min. The reaction was stopped by filtering off the gel on a Buchner funnel and washing with cold 0.1 M $NaHCO_3$ buffer (pH 10). Saturated L-tyrosine solution in the same buffer was coupled to sepharose-4B-L-tyrosine activated with CNBr. The reaction was completed by stirring for 90 min. In order to remove excess of L-tyrosine from the sepharose-4B-L-tyrosine gel, the mixture was washed with distilled water. The hydrophobic gel was obtained by diazotization of 1-naphthylamine and coupling of this compound to the sepharose-4B-L-tyrosine. The pH was adjusted to 9.5 with 1 M NaOH and, after gentle stirring for 3 h at room temperature, the coupled red sepharose derivative was washed with 1l of water and then 200 mL of 0.05 M Tris-sulfate (pH 7.5) [17]. The column (1.0 cm diameter × 5.0 cm length) was pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.8) including 1 M $(NH_4)_2SO_4$ before loading the enzyme solution. The enzyme was eluted using a linear gradient of 1.0–0.0 M $(NH_4)_2SO_4$ in the same buffer at a flow rate of 30 mL/h; 1 mL fractions were collected. The proteins containing the highest β-glucosidase activity were combined and used as purified enzyme for subsequent studies after confirming homogeneity by gel electrophoresis.

2.5. β-Glucosidase assay and protein determination

During enzyme extraction and purification, β-glucosidase activity was routinely determined using *para*- and *ortho*-nitrophenyl-β-D-glucopyranosides (*p*-NPG and *o*-NPG) as substrates. Appropriately diluted 70 μL of enzyme solution in 50 mM sodium acetate, pH 5.5 and 70 μL of substrate were mixed in the wells of a 96-well microtiter plate in quadruplicate. After incubation at 37 °C for 30 min, the reaction was stopped by adding 70 μL of 0.5 M Na_2CO_3, and the color that developed as a result of *p*-/*o*-nitrophenol liberation was measured at 410 nm. Enzyme activity was expressed as μmol *p*-/*o*-nitrophenol formed per minute in the reaction mixture under these assay conditions. Activity assay (Zymography) was carried out for detection of β-glucosidase activity after native-PAGE under non-denaturing conditions using 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) as a substrate.

Protein concentrations were determined [18] using bovine serum albumin (BSA) as the standard.

2.6. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were fractionated on 12% SDS-PAGE gels [19] using a Minigel system (Bio-Rad Laboratories, USA). Gels were fixed, stained with Coomassie brilliant blue R-250 (Sigma), and destained using standard methods to detect protein bands. For the detection of β-glucosidase activity in a non-denaturing PAGE, the enzyme solution was loaded onto 6% native polyacrylamide gel. After electrophoresis, the gel was equilibrated in two changes of 50 mM sodium acetate buffer, pH 5.5, for 15 min each, and then incubated with the substrate (4-MUG) for 15 min at 37 °C. The band corresponding to the enzyme activity was observed and photographed under UV light.

2.7. Determination of pH and temperature optimum

The effect of varying the pH on olive β-glucosidase activity was examined using 25 mM sodium acetate (3.0–5.8), citrate-phosphate (3.0–7.0) and phosphate (6.0–8.5) buffers. For temperature optimum determination, the enzyme and substrate *p*-NPG solution mixtures were assayed in the temperature range 4–65 °C for 30 min.

2.8. In vitro inhibition studies and determination of kinetic parameters

Various final concentrations of *p*-NPG (0.78–20 mM) and *o*-NPG (0.78–20 mM) were used to estimate the kinetic parameters K_m and V_{max}. Inhibition experiments were performed using *p*-NPG as substrate and different final concentrations of δ-gluconolactone, glucose and some metal ions as possible inhibitors. A double reciprocal Lineweaver–Burk plot was used to calculate the parameters. The activity of β-glucosidase for six different concentrations of each inhibitor was determined by regression analysis. Results are expressed a %; β-glucosidase activity in the absence of an inhibitor was taken as 100%. The inhibitor concentration that reduces the enzymatic activity by 50% (IC_{50} values) was determined from the plots (Figs. 5B and 6B).

3. Results and discussion

An olive (*O. europaea* L.) β-glucosidase was purified from crude extracts of olive acetone powder to apparent homogeneity by salting out with ammonium sulfate and using the specifically designed sepharose-4B-L-tyrosine-1-napthylamine hydrophobic interaction chromatography. Upon fractionation of the β-glucosidase active fractions with ammonium sulfate, 72% of the activity was obtained in the fraction saturated with 50% ammonium sulfate. This step removed the greater part of the contaminants and decreased total protein amount from 201 to 18.5 mg. The precipitate with β-glucosidase activity was dissolved and saturated with 1 M ammonium sulfate, to improve its binding efficiency, before applying to the sepharose-4B-L-tyrosine-1-napthylamine column. Fig. 1 shows the typical elution pattern of the enzyme activity on

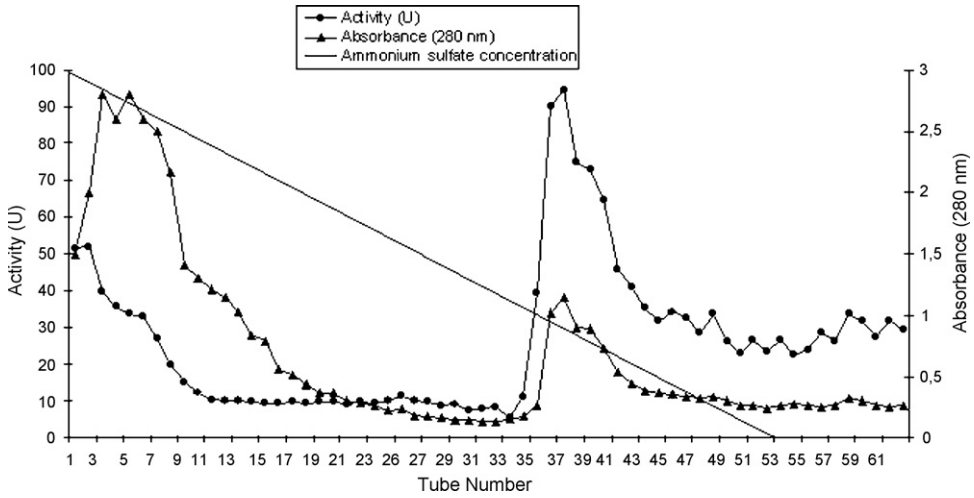

Fig. 1. Purification of an olive (*Olea europaea* L.) fruit β-glucosidase by hydrophobic interaction chromatography. The enzyme activity and total protein concentrations were determined, from all fractions collected, as described in Section 2. The enzyme activity was expressed as μmol of *p*-/*o*-nitrophenol liberated per minute in the reaction mixture under the assay conditions.

this hydrophobic column. The enzyme activity and total protein concentrations were determined for all fractions collected. The fractions with the highest β-glucosidase activity and the relatively lower protein contents were pooled. This newly developed hydrophobic interaction chromatography purified the enzyme from remaining contaminants to apparent homogeneity, retaining 75% of the activity from the previous step, therefore further purification steps were not required. The β-glucosidase was purified 154.9 fold with an overall enzyme yield of 53.9% and a specific activity of 254.9 U/mg (Table 1). The fold purification and the enzyme yield with the above procedure were higher than with previous β-glucosidase purification studies from different plant sources. Romero-Segura et al. [16] reported the purification of the enzyme with 8 folds purification factor and an enzyme yield of 8.3%. The enzyme from fresh leaves of tea plants was purified 47.7 fold by Li et al. [20]. The purification factor and the enzyme yield values of β-glucosidase from apple seed, vanilla bean and soybean have been reported as 46.1 fold and 12.8%, 7.2 fold and 8.4%, and 20 fold and 20%, respectively [21–23].

Employing the least number of sequential steps (two in this case) is important in obtaining maximum enzyme yield. Also, the structure and characteristics of matrix and ligand of hydrophobic interaction chromatography are critically important. In our study 1-napthylamine, which is a hydrophobic group, was added to sepharose-4B gel matrix with the extension of L-tyrosine arm. Having an aryl structured hydrophobic 1-napthylamine in the hydrophobic gel have been shown to increase the purification factor of the enzyme. Since aryl ligands have hydrophobic and aromatic characters, our results also suggest that the enzyme shows both hydrophobic and aromatic interactions with these residues.

SDS-PAGE analysis of the purified enzyme showed the presence of a single band with an apparent molecular mass of ca. 65 kDa, when stained with Coomassie brilliant blue (Fig. 2A). The estimated subunit molecular mass of the protein is similar to β-glucosidases from various plant sources, e.g. 64 kDa from orange (*Citrus sinensis* var. Valencia) fruit tissue [24], 68 kDa from ripe sweet cherry (*Prunus avium*) fruit [25] and 62 kDa from *Sorghum bicolor* seedlings [26].

In order to confirm the activity data of native protein with spectrophotometric assays, native-PAGE zymogram assay was performed. The result showing an activity band of beta-glucosidase is shown in Fig. 2B.

The pH optimum for β-glucosidase activity was 5.5 (Fig. 3), and the enzyme retained over 50% of the original activity between pH 4.0 and 5.7. This pH optimum is similar to that previously determined for β-glucosidases from various plant sources [16,20,29].

The enzyme displayed maximal activity at 42 °C (Fig. 4), which is lower than for some plant β-glucosidases that show the highest enzymatic activity at around 50 °C [20,35,36]. Increased catalytic activity at higher temperatures, e.g. 50 °C, is not physiologically significant because activity is lost due to thermal denaturation in a very short incubation time [20,29].

The reaction kinetics of the purified β-glucosidase was determined from Lineweaver–Burk plots with artificial substrates *p*-/*o*-nitrophenyl-β-D-glucopyranosides (*p*-/*o*-NPG). The enzyme was effectively active on *p*-/*o*-NPG with K_m values of 2.22 and 14.11 mM and V_{max} values of 370.4 and 48.5 U/mg, respectively. Affinity of the enzyme for *p*-NPG was considerably higher than for *o*-NPG. The higher β-glucosidase affinities for *p*-NPG have also been reported from vanilla bean [22], soybean [23], leaves of tea plant [20] and orange fruit tissue [24].

The inhibition kinetic experiments of the enzyme were performed using *p*-NPG as substrate, while δ-gluconolactone and glucose as inhibitors. The enzyme was inhibited by both potential

Table 1
Purification of β-glucosidase from olive (*Olea europaea* L.) fruit.

Step	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude extract	201.15	1.65	100	–
Ammonium sulfate	18.57	12.87	72.2	7.82
Hydrophobic chromatography	0.7	254.9	53.9 ([a]8.3, [c]12.8, [d]8.4, [e]20)	154.9 ([a]8.1, [b]47.7 [c]46.1, [d]7.2, [e]20)

[a] Ref. [16].
[b] Ref. [20].
[c] Ref. [21].
[d] Ref. [22].
[e] Ref. [23].

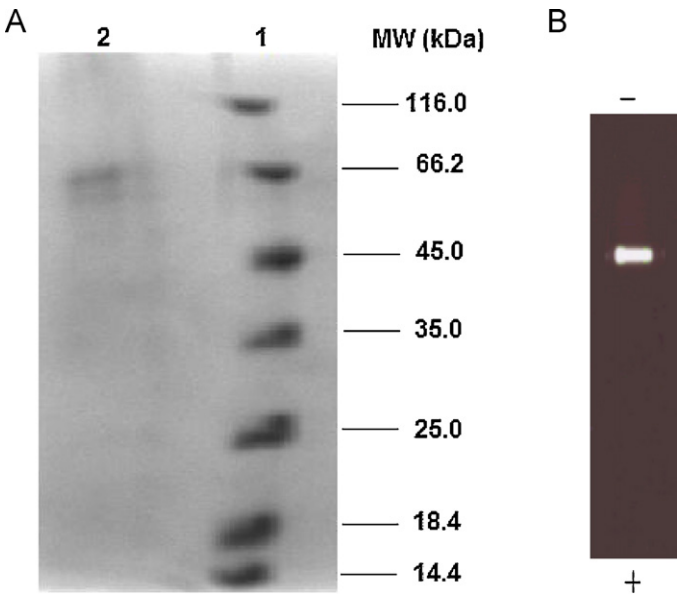

Fig. 2. (A) SDS-PAGE of the purified β-glucosidase from olive (*Olea europaea* L.) fruit. The enzyme was electrophoresed at pH 8.3 on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lane 1: molecular weight standards (β-galactosidase, 116 kDa; bovine serum albumin, 66.2 kDa; egg albumin, 45 kDa; lactate dehydrogenase, 35 kDa; Rease Bsp981 (*E. coli*), 25 kDa; β-lactoglobulin, 18.4 kDa; lysozyme, 14.4 kDa); lane 2: purified β-glucosidase. (B) Native-PAGE (6%) gel zymogram of β-glucosidase developed with the fluorogenic substrate 4-MUG.

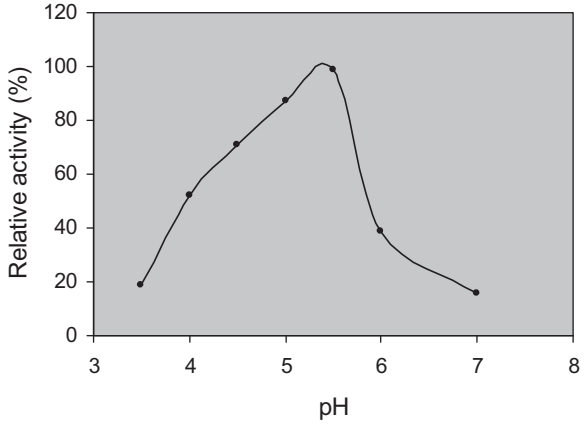

Fig. 3. Effect of pH on activity of purified olive (*Olea europaea* L.) fruit β-glucosidase.

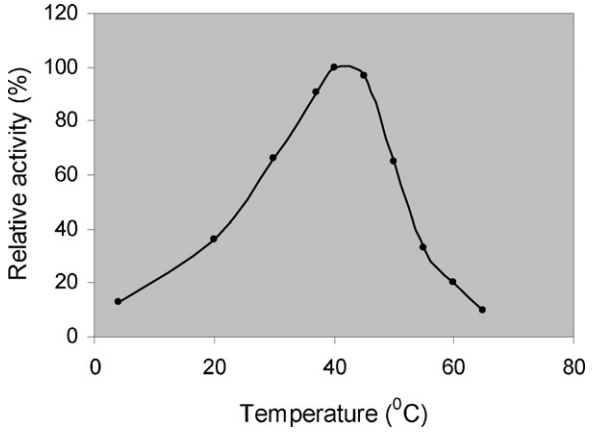

Fig. 4. Effect of temperature on activity of purified olive (*Olea europaea* L.) fruit β-glucosidase.

inhibitors investigated. δ-Gluconolactone was the more effective (competitive) inhibitor of the enzymatic activity with K_i value of 0.016 mM (Fig. 5A) and IC_{50} of 0.23 mM (Fig. 5B). This strong inhibitory effect of δ-gluconolactone is in agreement with previous reports regarding the inhibition of β-glucosidases from various plant sources [10,22,27]. The inhibition kinetics of β-glucosidases from several plant and especially microorganism sources have been extensively studied using glucose as an inhibitor, since glucose inhibition of β-glucosidases by glucose is undesirable if the enzymatic hydrolysis of cellulose is performed as an industrial process. β-Glucosidases from rice, *Arabidopsis thaliana* and vanilla bean were not inhibited by glucose at concentrations up to 2 M [22,28,29], while the enzyme from orange fruit was effectively inhibited by lower concentrations [24]. Highly glucose tolerant β-glucosidases have been reported from yeasts *C. sake*, *P. etchellsii*, *D. vanrijiae*, and *C. peltata* with K_i values of 0.2, 0.3, 0.44, and 1.4 M, respectively [30–33]. According to Saha and Bothast [34], β-glucosidase activities from some yeast strains were even stimulated by glucose. However, most microbial β-glucosidases are strongly inhibited by glucose with the inhibition constants ranging from 0.6 to 10 mM [13]. Interestingly, the olive fruit β-glucosidase activity was also inhibited competitively by glucose with K_i value of 6.4 mM (Fig. 6A) and IC_{50} value of 105.5 mM (Fig. 6B) towards *p*-NPG as substrate. This result is in agreement with the report by Cameron et al. [24] from *Citrus sinensis* var. Valencia fruit tissue.

The effect of various metal ions on the purified enzyme was examined (Table 2). Of the ions tested, only the Fe^{2+} increased the activity while Cd^{2+}, Pb^{2+}, Cu^{2+}, Ni^{+}, and Ag^{+} exhibited different levels and types of inhibitory effects. Cameron et al. [24] also reported that 1 mM $FeCl_3$ did not show an inhibitory effect

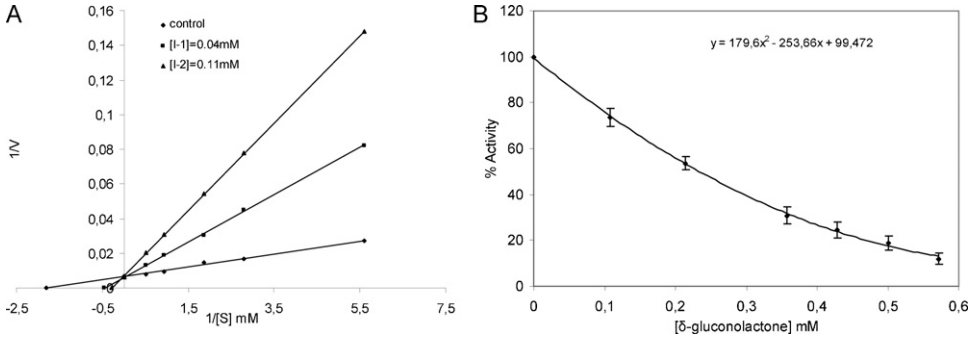

Fig. 5. Inhibition of the purified β-glucosidase from olive (*Olea europaea* L.) fruit by δ-gluconolactone. (A) Lineweaver–Burk plot with various substrates (*p*-NPG) and inhibitor (δ-gluconolactone) concentrations for determination of K_i and inhibition type. The intercept of plots indicates competitive inhibition for δ-gluconolactone. (B) Activity (%) curve of the β-glucosidase in the presence of different δ-gluconolactone concentrations.

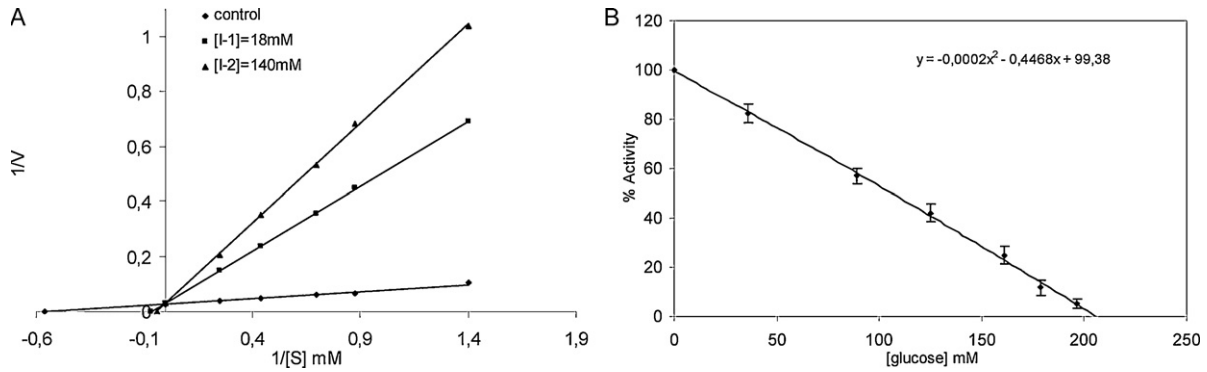

Fig. 6. Inhibition of the purified β-glucosidase from olive (*Olea europaea* L.) fruit by glucose. (A) Lineweaver–Burk plot with various substrates (*p*-NPG) and inhibitor (glucose) concentrations for determination of K_i and inhibition type. The intercept of plots indicates competitive inhibition for glucose. (B) Activity (%) curve of the β-glucosidase in the presence of different glucose concentrations.

Table 2
Effect of some metal ions on β-glucosidase activity from olive (*Olea europaea* L.) fruit.

Substance	K_i (mM)	Inhibition type	IC_{50} (mM)
[a] Fe^{2+}	–	–	–
Cd^{2+}	$4.29 \times 10^{-4} \pm 0.94 \times 10^{-5}$	Noncompetitive	$0.38 \times 10^{-4} \pm 0.002 \times 10^{-4}$
Pb^{2+}	$1.26 \times 10^{-2} \pm 5.68 \times 10^{-3}$	Uncompetitive	$5.3 \times 10^{-3} \pm 0.12 \times 10^{-3}$
Cu^{2+}	$2.26 \times 10^{-4} \pm 1.48 \times 10^{-4}$	Competitive	$6.1 \times 10^{-4} \pm 0.02 \times 10^{-4}$
Ni^{+}	$1.04 \times 10^{-4} \pm 6.98 \times 10^{-5}$	Competitive	$0.63 \times 10^{-4} \pm 0.004 \times 10^{-4}$
Ag^{+}	$3.21 \times 10^{-3} \pm 1.89 \times 10^{-4}$	Uncompetitive	$3.34 \times 10^{-3} \pm 0.17 \times 10^{-3}$

[a] Increase in enzyme activity.

on the β-glucosidase activity from *Citrus sinensis* var. Valencia fruit tissue. Cadmium was found to be as a strong noncompetitive inhibitor with K_m and IC_{50} values of 4.29×10^{-4} and 0.38×10^{-4} mM, respectively. Cu^{2+} was an effective competitive inhibitor with K_m and IC_{50} values of 2.26×10^{-4} and 6.1×10^{-4} mM, respectively. The inhibitory effect of Cu^{2+} on β-glucosidase activities have also been reported in studies on the enzyme from other plant sources such as olive, apple seed and vanilla bean [16,21,22]. Copper compounds are commonly used, especially copper sulfate, copper hydroxide, copper oxychloride and copper calcium sulfates as fungicides in agriculture. These results suggest that, the compounds containing copper ions can be considered as effective inhibitors of β-glucosidase activities. Ni^{+} was determined as a strong competitive inhibitor on the enzyme activity with K_m and IC_{50} values of 1.04×10^{-4} and 0.63×10^{-4} mM, respectively. Odoux et al. [22] showed that the vanilla bean β-glucosidase was also inhibited by Ni^{+} at 1 mM concentration. K_m and IC_{50} values for Pb^{2+} were determined as 1.26×10^{-2} and 5.3×10^{-3} mM, respectively. The inhibition type was uncompetitive since Pb^{2+} binds on the enzyme–substrate complex and is an effective inhibitor. Gueguen et al. [31] reported that 10 mM Pb^{2+} completely inhibited β-glucosidase activity from a yeast *Zygosaccharomyces bailli*. Silver ion showed an uncompetitive inhibition effect on the enzyme activity with K_m and IC_{50} values of 3.21×10^{-3} and 3.34×10^{-3} mM, respectively. This result is in agreement with published reports that Ag^{+} also showed inhibitory effects on the β-glucosidase activities from olive, apple seed, vanilla bean and soybean [16,21–23].

In conclusion, the present study has confirmed the effectiveness of the new purification procedure for β-glucosidase from olives (*O. europaea* L.). The enzyme was purified to apparent homogeneity in only two sequential steps and this is an important factor in obtaining the higher protein yield, by salting out with ammonium sulfate and using sepharose-4B-L-tyrosine-1-napthylamine hydrophobic interaction chromatography. The enzyme obtained from olives was characterized.

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