Increasing Antioxidant Activity in Food Waste Extracts by β-Glucosidase

Running head: Producing Potent Antioxidants by β-glucosidase

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SUMMARY

Research background. Food by-products such as onion peels and olive leaves are rich in bioactive compounds applicable as natural and low-cost sources of antioxidants. Still, these compounds mainly exist in glycosylated form. Often, hydrolysis of glycoside compounds increases their antioxidant activity and health benefits. However, not many studies have been done concerning the β-glucosidases effect, specifically from A. niger, on glycosylated compounds within these by-products. Also, changes in the antioxidant activity of the mentioned by-products under the effect of β-glucosidase have not been reported yet. Therefore, this study considered the effect of Aspergillus niger β-glucosidase on glucoside compounds and the antioxidant activity of onion peel and olive leaves extracts.
Experimental approach. The antioxidant activity of the extracts was assayed by DPPH and FRAP tests. Also, glucose, total phenolic, and flavonoid content were measured. Moreover, TLC and HPLC analyses were applied before and after the enzymatic hydrolysis.

Results and conclusions. The obtained results showed an increase in the extract antioxidant activity after treatment. Also, an enhancement was observed in the extracts glucose content by β-glucosidase. The TLC and HPLC results showed the β-glucosidase efficacy to hydrolyze quercetin glucosides in onion peel extract, and the quercetin content was increased from (0.479±0.04) mg/mL in the untreated extract to (1.262±0.027) mg/mL in the treated extract (0.5 % m/V) after 3 h enzymatic hydrolysis at 45 °C. Also, the content of quercetin-3-O-glucoside was increased considerably from (0.0018±0.0001) to (0.054±0.009) mg/mL following enzyme treatment. Moreover, oleuropein in olive leaves extract (1 % m/V) was hydrolyzed completely from (0.382±0.016) to 0 mg/mL by β-glucosidase for 24 h at 50 °C.

Novelty and scientific contribution. This study showed that A. niger β-glucosidase, as a stable enzyme, hydrolyzed quercetin and oleuropein glycosides in onion peel and olive leaves extracts. Thus, A. niger β-glucosidase is a good candidate for processing the food waste and extracting valuable bioactive compounds. Also, the treated extracts with higher antioxidant and biological activity, and without bitterness can be applicable as potent, natural, and cost-effective antioxidants in the food industry.

Keywords: antioxidant activity; β-glucosidase; oleuropein; quercetin; food waste

Abbreviations: DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: Ferric reducing antioxidant power; HPLC: High-performance liquid chromatography TLC: Thin-layer chromatography

INTRODUCTION

Due to increasing people's awareness about fruits and vegetables' health benefits, consuming these products has greatened. Nevertheless, many fruits and vegetables are processed to separate the desired ingredients. During the process of these crops, enormous amounts of waste are generated. The resulting by-products from plants processing are generally used as raw materials for animal feeding or agricultural uses. While they are rich in bioactive components applicable in different industries as the natural, economical, novel, and low-cost sources of antioxidants, dietary fiber, organic acids, enzymes, pectin, food additives, essential oils, etc. (1).
The onion peel is one of these by-products. The outer scales, peel, and onion roots are deleted and regarded as waste during food processing. Various onions (white, red, yellow) are rich in flavonols such as quercetin (mainly), kaempferol, tannins, and organosulfur components. Quercetin, quercetin-4-O-glucoside, and quercetin-3,4-O-diglucoside are major forms of quercetin in onions (2). Quercetin is a food additive, colorant, and raw material for the pharmaceutical, cosmetic, and fine chemical industries (3). The onion peel contains more quercetin derivatives than the onion and other vegetables and fruits. Gorinstein et al. (4) declared that quercetin in onion peel was 48 fold higher than the flesh. So, the onion peel is a source of bioactive compounds applicable as economic material to extract these precious ingredients.

Also, olive leaves are inexpensive by-products of olive trees and olive oil mills. Oleuropein is the main phenolic compound in olive leaves and fruit and an agent of bitterness in olives. It is an ester of hydroxytyrosol, elenolic acid, and glucose (5). Oleuropein aglycone is the oleuropein hydrolysis yield. Different potential applications have been antioxidant, bacteriostatic, anticancer, and antiviral (6). Also, oleuropein aglycon hydrolysis produces hydroxytyrosol, a potent antioxidant without bitterness. The production of these valuable compounds from renewable materials makes olive waste reused.

Although the obtained by-products from fruits and vegetables contain phenolic and flavonoid compounds, including high antioxidant activity, these compounds mainly exist in glycosylated form. Often, the non-sugar form of these substances has been shown to have higher antioxidant activity than their glycosides. Also, hydrolysis of glycosylated components increases the extraction yield of their aglycones. Moreover, enzymatic hydrolysis is preferred to chemical because the enzyme selectively acts on desired compounds, produces lower secondary metabolites, other precious ingredients are not damaged, and decreases environmental problems. Thus, enzymatic hydrolysis can be an alternative to chemical methods.

β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) are biological macromolecules. These enzymes break glycosidic bonds in alkyl- and aryl-β-D-glycosides and various oligosaccharides (7). One of the technological applications of β-glucosidases is the liberation of phenolic compounds (aglycone form) with antioxidant activity from fruit and vegetable residues. In this manner, Pyrococcus furiosus β-glucosidase has been used for hydrolysis of flavanone glycosides in grapefruit peel, grapefruit pulp, and orange peel extracts (8). The recombinant thermostable β-glucosidases from Thermotoga neapolitana have been converted quercetin glucosides to quercetin in anion waste extract (9). The recombinant β-glucosidase from Myceliophthora thermophila and β-glucosidases from almonds have been hydrolyzed oleuropein from olive leaf extracts (10,11).
Moreover, β-glucosidase from *Aspergillus niger* has been hydrolyzed this compound in olive mill wastewater (12).

However, low studies have been done concerning the β-glucosidases effect, specifically from *A. niger* (as a food-grade and inexpensive microorganism for enzyme production with application potential on an industrial scale) for hydrolysis glycosylated compounds in onion peel and olive leaves extracts. Moreover, this enzyme's effect on the antioxidant activity of the mentioned by-products has not been reported. This study investigated the β-glucosidase efficacy from *A. niger* on glycoside components and antioxidant activity of onion peel and olive leaves extracts due to valuable ingredients in these food residues, including potential application in food as natural and cost-effective antioxidants. The effect of enzyme doses and reaction time with β-glucosidase on antioxidant activity in the onion peel and olive leaves extracts were considered using DPPH and FRAP tests. Also, the enzyme efficacy for hydrolysis of glycoside compounds was assayed by measuring glucose and TLC and HPLC analysis before and after the enzyme treatment.

**MATERIALS AND METHODS**

*Materials*

The *A. niger* β-glucosidase from previous work was used (13). The fungus was isolated from farm soil, and after the determination of its genus and species was applied. The β-glucosidase purification was performed by two stages of ion-exchange chromatography. Onion was purchased from the local market. Olive leaves were separated from olive trees in local orchards in Kermanshah province. The quercetin, quercetin-3-O-glucoside, Trolox, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (USA, St. Louis). Oleuropein was supplied from Cayman Chemical Company (USA, Ann Arbor). Other chemical materials were of analytical grade and prepared from Merck (Germany, Darmstadt).

*The extracts preparation*

According to Lee *et al.* (14), the onion peel extract was prepared with modifications. At first, aqueous ethanol (20 mL of solution 60 %) was added to 1g of dried and powdered red onion peel, and the mixture was stirred at room temperature for 60 min. Then, the mixture was filtered by a Büchner funnel, including Whatman No. 2 filter paper, again 20 mL of solvent added to the residue. The extraction was repeated for the other 30 min. After filtration, the solvent was removed at 40 °C by a rotary evaporator (SB-1200, EYELA, Tokyo, Japan) under a vacuum.
The extract preparation was performed for olive leaves according to Yateem et al. (15) with modifications. Primarily, the olive leaves were washed, dried in an oven at 50 °C, and powdered. Then, 100 mL of aqueous ethanol 80 % was added to 10 g of olive leaf powder, and the mixture was stirred for 4 h at room temperature. After filtration by the Whatman filter paper, the ethanol was evaporated at 40 °C by a rotary evaporator under a vacuum. Then, some water was added to the extract, and it was centrifuged (2-16PK, Sigma, Berlin, Germany) at 6500 rpm for 5 min for the separation of chlorophyll and other water-insoluble compounds. Next, almost 2 mL of dichloromethane was added to the extract, and after mixing for 1 min, the mixture was centrifuged as mentioned. Finally, the water evaporated as declared, and the extracts were stored at 4 °C until analysis time.

The effect of β-glucosidase on antioxidant activity of onion peel and olive leaves extracts

DPPH test

For assay of DPPH radical scavenging activity, the extracts solution in concentrations 0.02 % m/V (onion peel), and 0.1 % m/V (olive leaves) were prepared in 100 mM acetate buffer (pH 5). The anion peel stock solution (0.5 % m/V) was prepared in acetate buffer containing 30 % V/V ethanol. Then, 100 µL of β-glucosidase in the same buffer (0.1 µg/mL in reaction medium) was added to 400 µL of each extract, and the reaction was done at time intervals of 15 min for 90 min at 60 °C. Also, various enzyme doses at a range of 0-1 µg/mL were reacted with the extracts in a total volume of 500 µL at 60 °C for 15 min. The enzyme was inactivated by heating the extracts at 90 °C for 5 min. The DPPH test was carried out according to Brand-Williams et al. (16) with slight modifications. The DPPH stock solution was prepared by solving 4.8 mg of DPPH in 20 mL methanol and was stored at −20 °C until analysis time. For analysis, the DPPH solution was diluted with methanol 5.5 times. Then, 950 µL of this solution reacted with 50 µL of the extract for 1 h in the dark. The blank sample was prepared by adding 50 µL of acetate buffer to 950 µL of DPPH reagent. The absorbance read at 515 nm by spectrophotometer UV-VIS (Lambda 25, Perkin Elmer, Waltham, USA) and % radical scavenging was calculated as follows:

% Radical scavenging = \((A_b - A_S)/A_b \times 100\)

where \(A_b\) and \(A_s\) are the absorbance of the blank and sample, respectively.

Ferric reducing antioxidant power (FRAP) test

The FRAP test is based on enhancement in absorbance at 595 nm due to TPTZ-Fe^{3+} complex formation. For this test, the extracts solution was prepared at concentrations 0.05 % m/V (olive leaves)
and 0.01 % m/V (onion peel). As stated in the previous section, the effect of enzyme concentration and the reaction time was assayed on the extracts reducing power. The FRAP test was performed according to Benzie and Strain (17). The stock reagents include acetate buffer (300 mM, pH 3.6), FeCl₃. 6H₂O (20 mM solution), and 2,4,6- tripyridyl-s-triazine (TPTZ, 10 mM in 40 mM HCl) were prepared. The working reagent was prepared as fresh by combining 2.5 mL TPTZ, 2.5 mL FeCl₃. 6H₂O, and 25 mL of acetate buffer solutions and warmed to 37 °C before using. The extract (50 µL) was added to the working reagent (950 µL), and incubation was performed at 37 °C for 30 min in the dark. The absorbance read at 595 nm. The standard curve was provided by concentrations 0-500 µM of the Trolox standard, and antioxidant activity stated as µM of Trolox equivalent.

**The measurement of total phenolic and flavonoid content in onion peel and olive leaves extracts**

The total phenol was measured using the reagent of Folin–Ciocalteu according to Roesler et al. (18) with modifications before and after the β-glucosidase effect on the extracts. For this mean, 400 µL of olive leaf (1 % m/V) and onion peel (0.5 % m/V) extracts were reacted with β-glucosidase at concentrations of 1.5 and 2 µg/mL in a total volume of 500 µL, respectively. The extracts' reaction time with β-glucosidase was 24 h at 50 °C for olive leaves and 3 h at 45 °C for onion peel. The enzyme was inactivated by heating the extracts at 90 °C for 5 min. The extracts (100 µL) were mixed with the Folin–Ciocalteu reagent (500 µL of 1:10 diluted), and the mixtures were incubated at 25 °C for 5 min. Then, 400 µL of 5 % Na₂CO₃ was added, and again incubation was done in a water bath (ONE-29, Memmert, Schwabach, Germany) at 50 °C for 5 min. Next, the absorbance read at 765 nm after cooling the samples to room temperature. A standard curve was prepared using gallic acid (0-80 µg/mL) to estimate total phenol content (TPC). The TPC was calculated as mg gallic acid equivalent per gram of the dried powders of onion peel and olive leaves.

According to Kosalec et al. (19), the total flavonoid content was determined before and after the β-glucosidase effect on the extracts, as mentioned above. Initially, 250 µL of the extract was poured into a test tube. Then, it was mixed with 750 µL of 96 % ethanol, 50 µL of 10 % AlCl₃ (m/V), 50 µL of 1M sodium acetate, and 1.4 mL of distilled water. After incubation for 30 min at room temperature, the absorbance read at 415 nm. A standard curve was provided using quercetin (0-100 µg/mL) to determine the extracts' flavonoid content. The total flavonoid content was defined as mg of quercetin equivalent per gram of the dried powders of onion peel and olive leaves.

**Glucose measurement**
The glucose was measured by Glucose (HK.) Assay Kit (Greiner Diagnostic GmbH, Germany, Bahlingen). The β-glucosidase effect was investigated on the extracts, as stated in measuring total phenolic and flavonoid content. After inactivation of the enzyme, the absorbance of the extracts without enzyme treatment and treated ones read at 340 nm. The glucose content (mg/dL) was determined using kit manufacturer instructions.

Thin-layer chromatography (TLC) analysis

The β-glucosidase effect on the extracts investigated, as stated in the section on the measurement of total phenolic and flavonoid content. After the enzyme's inactivation, The extracts were analyzed by loading 5 μL of the reaction mixture on TLC plates (silica gel 60 F254, 20 × 20 cm). Glucose, quercetin, quercetin-3-O- glucoside, and oleuropein were used as standards. The separation was done by the solvent composition of butanol, acetic acid, and water (3,1,1). The spots were visualized by UV light at 254 nm and dipping plates in a methanol solution containing 5 % (V/V) sulfuric acid and heating at 120 °C for 10 min (20). The semi-quantitative analysis of the TLC plate was performed using Image J software (http://rsb.info.nih.gov/ij/index.html) (13,21). The TLC plate was analyzed as vertical for lines related to the extracts before and after enzyme treatment, and results were stated as the percent of pick area for desired compounds (quercetin and oleuropein) (UV light). Also, the TLC plate was analyzed as horizontal for a semi-quantitative comparison of glucose in the extracts before and after treatment. Results were stated as the percent of glucose peak area (H₂SO₄).

High-performance liquid chromatography (HPLC) analysis

The β-glucosidase effect on the extracts and oleuropein standard was investigated as declared in the section on measuring total phenolic and flavonoid content. Oleuropein standard was applied at a concentration of 1 mg/mL. The total volume of the reaction mixture for samples and standard was 500 μL. 500 μL of ethanol was added to each extract and standard at the end of the reaction time. The standards of oleuropein, quercetin, and quercetin 3-O-glucoside prepared as 50 % (V/V) ethanol solutions in the concentration of 1 mg/mL. The standards and extracts were filtered and analyzed by an Agilent 1260 HPLC system (Agilent Technologies, CA, USA). The separation was done by the MZ Perfectsil C₁₈ column (250 × 4.6 mm × 5 μm) for onion peel extract. The analysis condition was according to Kim et al. (22). The mobile phase included water: formic acid (95:5, V/V) (A) and 100 % methanol (B). The binary gradient was applied as follows: 20-60 % B, 0-25 min; 60-100 % B, 25-25.1 min; 100-60 % B, 25.1-30 min; 60-20 % B, 30-30.1 min; and 20 % B, 30.1-35 min.
The flow rate and the injection volume were 1 mL/min and 10 µL, respectively. The absorbance read at 360 nm. According to Mazzei et al. (23), the olive leaf extract was analyzed. The water and acetonitrile (79:21) mixture was acidified with o-phosphoric acid up to pH 3 and used as a mobile phase in the same column. The flow rate and the injection volume were 1.2 mL/min and 5 µL, respectively. The compounds were detected at 280 nm, and ingredients identification in the extracts was performed by comparing their retention time with standards and literature data. Also, quercetin, quercetin-3-O-glucoside, and oleuropein content in the extracts were measured before and after β-glucosidase treatment using the related standards' calibration curves in range 0-1 mg/mL for quercetin-3-O-glucoside, 0-1 mg/mL for oleuropein, and 0-0.5 mg/mL for quercetin. Calculations was performed based on 0.5 % m/V onion peel and 1 % m/V olive leaves extracts.

Statistical analysis
Using ANOVA, the data analysis was done by SAS (version 9) (SAS Institute Inc., Cary, NC, USA) software (13,24). The significant differences were considered using the LSD test at p<0.05. The semi-quantitative analysis of the TLC plate was carried out by Image J software (http://rsb.info.nih.gov/ij/index.html) (13, 21).

RESULTS AND DISCUSSION
The effect of β-glucosidase on antioxidant activity of onion peel and olive leaves extracts

DPPH test
The effect of β-glucosidase treatment on the radical scavenging activity of the extracts is shown in Fig. 1. According to the figure, the extracts' antioxidant activity was enhanced with an increase in the enzyme concentration, but in olive leaves extract, the antioxidant activity remained constant at 0.25 μg/mL β-glucosidase and higher concentrations of the enzyme. The extracts' incubation time with β-glucosidase significantly affected DPPH radical scavenging, and antioxidant activity enhanced until 60 min. However, almost both extracts did not show a considerable increase in the antioxidant activity during 60-90 min (p<0.05). Totally, at the end of 90 min reaction with the enzyme, the percent of DPPH radical scavenging was 68.05, and 54.02 for onion peel and olive leaves extracts, respectively, which showed an increase of 1.41 and 1.16 times relative to initial scavenging activity in non-treated extracts. This enhancement is related to the hydrolysis of glycosidic compounds by β-glucosidase in the mentioned extracts and increasing free hydroxyl groups on the flavonoid ring (25).

Similarly, citrus by-products antioxidant activity increased with tannase, pectinase, cellulase,
and β-glucosidase treatment (26). A slight decrease in antioxidant activity was observed by the reaction of olive leaves extract with β-glucosidase at 50 °C for 24 h (data not shown) due to hydrolysis of oleuropein aglycon in the water medium. Similar results were obtained by Yuan et al. (27). They observed olive leaf extract scavenging activity decreased after hydrolysis of oleuropein by hemicellulase.

FRAP test

The FRAP test results showed increased antioxidant activity as µM of Trolox equivalent (TE) for both extracts after the enzyme treatment (Fig. 2). The antioxidant activity remained constant in both onion peel, and olive leaves extracts at concentrations of 0.5 and 0.25 µg/mL of β-glucosidase and higher enzyme concentrations. Also, the extracts' reaction time with β-glucosidase had a significant effect on the antioxidant activity. Among the treated extracts, the highest antioxidant activity was obtained for onion peel after 90 min (from 275.86 to 344.26 µM TE), followed by olive leaves (from 219.2 to 240.8 µM TE). The obtained results for the FRAP test agreed with the achieved ones by the DPPH test. Kim and Jang (28) observed a significant increase in mulberry leaf extract's antioxidant activity using β-glucosidase. Their study performed the antioxidant activity assay by oxygen radical absorbance capacity (ORAC) and cellular antioxidant capacity (CAC). The different results were obtained in the Cavia-Saiz et al. (29) study by treating grapefruit juice with naringinase enzyme treatment. They observed the lower antioxidant activity in the treated extract than in the fresh juice, while the antioxidant capacity increased by the ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) method. They declared this can be due to the loss of vitamin C during pasteurization of the extract, which causes a decrease in the extract's reducing power. In another study, antioxidant activity was increased in raspberry wastes by commercial enzymes cellulase, hemicellulase, and pectinase. The antioxidant activity was assayed by DPPH, ABTS, and FRAP tests (30). Also, in a recent study, we considered the effect of A. niger β-glucosidase on grapefruit peel extract. The data showed an increase of 2.09 times in the extract antioxidant activity after 90 min treatment with the enzyme (31).

The achieved data for antioxidant activity by FRAP and DPPH tests represent more bioactive and valuable extracts after the enzymatic hydrolysis that prone them to be applicable as inexpensive, robust, and natural antioxidant compounds in the food industry.

The measurement of total phenolic and flavonoid content in onion peel and olive leaves extracts

Before and after the enzymatic hydrolysis, the total phenolic and flavonoid content was
measured using gallic acid and quercetin as standards, respectively. The total phenol was slightly increased from (16.134±0.184) to (17.489±0.722) mg/g (dry olive powder) for olive leaves extract and from (40.752±1.071) to (41.688±0.612) mg/g (dry onion powder) for onion peel extract after enzyme treatment. Also, flavonoid content was increased after enzyme treatment from (2.313±0.068) to (2.655±0.031) mg/g for olive leaves extract and unchanged (16.293±0.475) mg/g for onion peel extract. However, this increase was not significant (p<0.05). Total phenol and flavonoid content must be constant before and after the enzyme treatment. After the enzymatic hydrolysis, only the glucose separates from the glycosylated compounds, producing their aglycon. Still, their total amount in the medium is not changed. Although, the content of each phenolic and flavonoid component alone may be increased or decreased after treatment.

Moreover, in the Folin–Ciocalteu method, the total phenol content is higher than the actual range of these components due to the reagent’s unselectivity. This reagent reacts with other reducing agents such as sugars (glucose) furthermore phenolic compounds (32). Also, the Folin reagent reacts with hydroxyl groups of phenols to form the blue dye. After the enzymatic hydrolysis, blue dye intensity increases due to the separation of glucose from the glycoside compounds and an increase in free hydroxyl groups on the flavonoid ring. Cavia-Saiz et al. (29) observed a 78 % increase in grapefruit juice’s total phenols after treatment by naringinase. Wang et al. (33) observed a significant increase in soluble phenols and flavonoid content by treating guava leaves extract using a mixture of ß-glucosidase and cellulase. In another report, the content of phenolic and flavonoid compounds in oilseeds cake extract of Borago officinalis, Oenothera biennis, and Nigella sativa increased after treatment ß-glucosidase, ß-glucanase, α-amylase, and their combinations (25). Ruviaro et al. (26) obtained similar data by treating citrus waste extract with tannase, pectinase, cellulase, and ß-glucosidase.

Glucose measurement

The glucose was measured according to the hexokinase kit instruction before and after the enzymatic hydrolysis. The phenolic and flavonoid compounds in the onion peel and olive leaf extracts interfere with glucose measurement by conventional methods such as the DNS and glucose-oxidase/peroxidase kits. Thus, the glucose content was measured by the hexokinase kit. The glucose content was increased significantly in onion peel extract from (222,16±1.16) to (247,66±0.33) mg/dL and in olive leaves extract from (26.95±0.74) to (76.65±0.55) mg/dL after the ß-glucosidase treatment (2.8 and 1.11 fold for olive leaves and onion peel extracts, respectively). The high glucose content in olive leaves extract after enzymatic treatment is related to oleuropein’s hydrolysis as the main
phenolic compound in olive leaves. The increase in glucose content in onion peel extract is associated with the hydrolysis of quercetin glucosides by β-glucosidase. Similar results were observed in the study of Ratz-Lyko et al. (25).

Thin-layer chromatography (TLC) analysis

The results of the TLC analysis are shown in Fig. 3 and Fig. 4. According to Fig. 3a, quercetin glycosides were hydrolyzed by treating the onion peel extract with β-glucosidase. As a result, quercetin spot density increased in onion peel extract after the enzymatic hydrolysis. The TLC plate analysis by Image J software (Fig. 3b) confirmed the quercetin increase in onion peel extract after the enzyme action (quercetin peak area: from 48.45 % to 84.51 %). Also, the extract treatment increased glucose spot density (glucose peak area: from 48.36 % to 51.64 %). Choi et al. (34) obtained similar data by treating onion skin waste with cellulase, xylanase, and pectinase. Also, the enzyme hydrolyzed oleuropein in olive leaves extract entirely (Fig. 4a), and as a result, the oleuropein spot disappeared in olive leaves extract after enzyme hydrolysis.

Moreover, glucose spot density was increased considerably in olive leaf extract after the enzyme treatment. The obtained data by Image J software confirmed complete hydrolysis of oleuropein and increasing glucose (glucose peak area: from 37.17 to 62.85 %) in treated olive leaves extract (Fig. 4b). Similar data was not found for the enzyme effect on olive leaf extract in the TLC analysis literature. It is the first report to analyze olive leaves and onion peel extracts on a TLC plate using Image J software.

High-performance liquid chromatography (HPLC) analysis

The HPLC analysis for onion peel extract showed a considerable increase in quercetin content after β-glucosidase treatment (Table 1). Also, the content of quercetin 3-glucoside increased significantly after the enzyme treatment. The enzyme did not hydrolyze the quercetin 3-glucoside standard (data not shown). Thus, other quercetin glucosides, including quercetin 4-glucoside and quercetin 3, 4-di-glucoside, were hydrolyzed by β-glucosidase. As a result, the peak with the retention time of 19.65 min in the sample chromatogram before treatment, according to the literature (9,35), is related to quercetin 4-glucoside, disappeared entirely after the enzymatic hydrolysis (data not shown). Similar results were observed in the mentioned literature for treated onion waste by Thermotoga neapolitana β-glucosidase. Moreover, β-glucosidase affected other glycosides in the onion peel extract.
In olive leaves extract, oleuropein (the main phenolic compound) was hydrolyzed completely by \( \beta \)-glucosidase within 24 h reaction at 50 °C (Table 1). As a result, the bitterness of the extract disappears after enzymatic treatment. Still, the oleuropein aglycon's pick did not appear in the sample chromatogram after treatment (data not shown). The oleuropein aglycon has poor water stability. Typically, 24 hours after production, it spontaneously converts to hydroxytyrosol and elenolic acid or by esterase. Also, in the treated sample, a pick with a retention time of 5.22 min appeared, according to the literature (10,36), related to hydroxytyrosol. After treatment by \( \beta \)-glucosidase, the exact pick was observed in the oleuropein standard chromatogram. It means spontaneous hydrolysis of oleuropein aglycon in the water medium.

Furthermore, the enzyme affected other glycosides in the olive leaves extract. The \( \beta \)-glucosidase from different sources, including Streptomyces sp (37), almond (5,38), and olive (39,40), has been used for oleuropein hydrolysis.

The results of different exams showed the efficacy of \( \beta \)-glucosidase for processing the mentioned by-products. According to the previous study (13), A. niger \( \beta \)-glucosidase is a stable enzyme with high storage stability, heat stability, pH stability, and stability against organic solvents. Therefore, it is a potential enzyme for food wastes processing and extraction and recovery of worth bioactive compounds without high costs. Moreover, hydrolysis of glycoside compounds into aglycone form increases their health benefits.

**CONCLUSIONS**

This study showed the efficacy of \( \beta \)-glucosidase for quercetin glycosides' hydrolysis in onion peel extract. Also, oleuropein (bitterness agent) in the olive leaf extract was hydrolyzed completely by \( \beta \)-glucosidase. As a result, the extract bitterness was eliminated by enzyme treatment. Different tests were in accordance, and antioxidant activity in onion peel and olive leaf extracts increased after the enzymatic hydrolysis. Thus, the hydrolysis by the \( \beta \)-glucosidase improved the mentioned extracts' bioactive properties, and the extracts with more potent antioxidant activity and desired flavor can be used as natural and low-cost antioxidants in the food industry. Moreover, the extraction yield of quercetin and hydroxytyrosol was enhanced by \( \beta \)-glucosidase, which prone it to apply as an alternative biological method instead of chemical processes to extract these worthy compounds. Also, the treatment of the onion peel and olive leaves extracts by the \( \beta \)-glucosidase provides the possibility of reusing these food wastes. As a result, \( \beta \)-glucosidase from A. niger (as a food-grade and inexpensive microorganism for enzyme production and applicable on an industrial scale), a stable
enzyme, has a high potential for by-products processing and extraction of bioactive compounds at a low cost, which is very important for the food industry.

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CONFLICT OF INTEREST

We do not have any conflicts of interest.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr.

AUTHORS’ CONTRIBUTION

F. Karami participated in data analysis and interpretation, performing the analysis and drafting the article. M. Ghorbani assisted in critical revision and final approval of the version to be published. A. Sadeghi Mahoonak also participated in the critical revision and final approval of the version to be published. A. Pourhossein assisted in performing the analysis. A. Bagheri also participated in performing the analysis. R. Khodarahmi assisted in designing the work, critical revision, and final approval of the version to be published.

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Fig. 1. The effect of enzyme concentration and reaction time with β-glucosidase on DPPH radical scavenging activity of onion peel (a, b) and olive leave (c, d) extracts. The data are an average of two replicates.
Fig. 2. The effect of enzyme concentration and reaction time with β-glucosidase on reducing power (FRAP) of onion peel (a, b) and olive leave (c, d) extracts. The data are an average of two replicates.
Fig. 3. Thin-layer chromatography (TLC) analysis (a) for considering the effect of β-glucosidase on glycoside compounds in onion peel extract; Lanes 1-5 are glucose, quercetin 3-O-glucoside, quercetin, the extract before enzyme treatment, and the extract after enzyme treatment, respectively. TLC plate visualized by UV light at 254 nm (left) and under H₂SO₄ (right). The vertical (left) and horizontal (right) analysis of the TLC plate before (lane 4) and after (lane 5) enzyme hydrolysis by Image J software (b)
Fig. 4. Thin-layer chromatography (TLC) analysis for considering the effect of β-glucosidase on glycoside compounds in olive leaves extract (a); lanes 1-4 are glucose, oleuropein, the
extract before enzyme treatment, and the extract after enzyme treatment. TLC plate visualized by UV light at 254 nm (left) and under H$_2$SO$_4$ (right). The vertical (left) and horizontal (right) analysis of the TLC plate before (lane 3) and after enzyme hydrolysis (lane 4) by Image J software (b)
Table 1. The results of HPLC analysis for onion peel and olive leaves extracts before and after treatment by β-glucosidase

<table>
<thead>
<tr>
<th>Extract</th>
<th>w(quercetin)/(mg/mL)</th>
<th>w(quercetin-3-O-glucoside)/(mg/mL)</th>
<th>w(oleuropein)/(mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion peel before treatment</td>
<td>(0.479±0.04)a</td>
<td>(0.0018±0.0001)a</td>
<td>nd</td>
</tr>
<tr>
<td>Onion peel after treatment</td>
<td>(1.262±0.027)b</td>
<td>(0.054±0.009)b</td>
<td>nd</td>
</tr>
<tr>
<td>Olive leaves before treatment</td>
<td>nd</td>
<td>nd</td>
<td>(0.382±0.016)a</td>
</tr>
<tr>
<td>Olive leaves after treatment</td>
<td>nd</td>
<td>nd</td>
<td>(0)</td>
</tr>
</tbody>
</table>

Data are means ± standard deviation of three replicate. The similar characters mean no significant difference at p<0.05. Nd: not determined.