Efficient bioconversion of oleuropein from olive leaf extract to antioxidant hydroxytyrosol by enzymatic hydrolysis and high-temperature degradation

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Abstract

Hydroxytyrosol (HT), a powerful antioxidant, clears free radicals and exhibits many biological activities. Because contents of HT are low in natural sources, bioconversion of oleuropein (OLE) to HT is of increasing interest. A biotechnological process was investigated to produce HT from OLE presented in olive leaf extract. Enzymatic hydrolysis using two cellulases with high *β-glucosidase* activity, Novozymes CTec2 and commercial cellulase KDN (Qingdao, People's Republic of China) was carried out at 50 °C for 12 H followed by raising the temperature to 90 °C for chemical hydrolysis. After 48 H of hydrolysis, an OLE degradation rate of 100% and a HT yield of 86–88% were achieved. These cellulases degrade OLE and release a glucose molecule. Chemical hydrolysis at a high temperature promotes the cleavage of ester bond and the formation of HT. This process has a promising alternative for production of HT comparing with acid hydrolysis which not only causes significant pollution to the environment but also makes difficult to the subsequent separation. Volume 65, Number 5, Pages 680–689, 2018

Keywords: *antioxidant, cellulase, enzymatic hydrolysis, hydroxytyrosol, oleuropein*

1. Introduction

The olive species (*Olea europaea* L.) are evergreen woody trees that have been cultivated for centuries. Although olive trees are famous worldwide for their oil, leaves, and fruit, 90% of trees are mainly cultivated only in Mediterranean countries, such as Spain, Greece, Italy, Tunisia, and others [1]. It is well known that abundant polyphenolic compounds reside in oil [2], fruits [3], and leaves [4] of olive trees; these bioactive compounds possess high value-added strong antioxidant and antimicrobial activities that have attracted growing interest from scientists in recent years.

Olive leaves are cheap by-products of olive tree cultivation and olive oil mills. Each year, 25 kg of leaves are removed per olive tree during tree pruning; collectively, the total weight of leaves across the industry comprises close to 10% of the total weight of harvested olives [5]. The polyphenolic compounds of olive leaves are primary secoiridoids, flavonoids, and simple phenolic compounds [6].

Oleuropein (OLE), a polyphenolic compound responsible for the bitter taste of olive oil and fruit, is an ester that consists of hydroxytyrosol (HT), glucose, and elenolic acid [7]. Figure 1 shows the schematic diagram of OLE degradation by acid hydrolysis. The chemical structure of OLE includes an ester bond and a glucosidic bond. Previous research has demonstrated that all parts of the olive tree contain OLE. Indeed, OLE is the most abundant polyphenol in olive leaves [8], with OLE content reaching 10%–17% of the dry mass of leaves.

Abbreviations: *BG, β-glucosidase; FPA, filter paper activity; HPLC, high-performance liquid chromatography; HT, hydroxytyrosol; IUPAC, International Union of Pure and Applied Chemistry; OLE, oleuropein; pNPG, p-nitrophenylnitrophenyl-β-D-glucopyranoside.*

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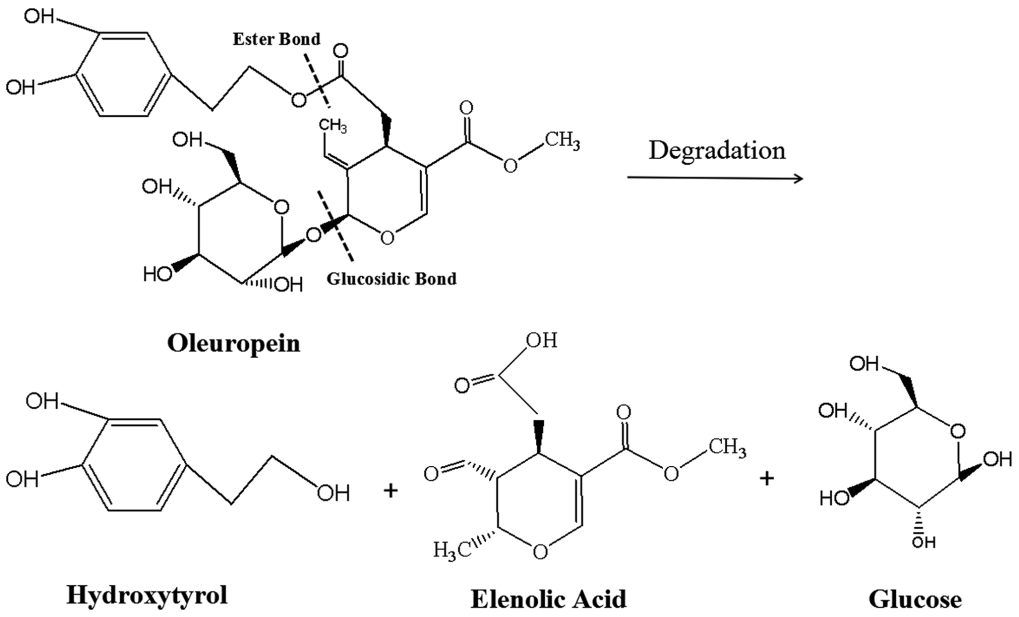

FIG. 1 *The schematic diagram of OLE degradation by acid hydrolysis.*

HT, which is also known as 3,4-dihydroxyphenylethanol, is one of the main products of OLE degradation and is one of the most powerful naturally derived antioxidants [9]. After evaluating the scientific evidence, the European Food Safety Authority deemed that HT is a protector of low-density lipoprotein from oxidative damage [10]. Recently, numerous *in vitro* studies and animal models have established that HT can prevent many diseases, due to its anticarcinogenic [11], anti-inflammatory [12], and antimicrobial [13] activities. In addition, protective activities have been described against high cholesterol [14], metabolic diseases [15], antigenotoxicity, cytotoxicity, and proapoptotic effects [11], oxidative stress [16], and digestive disorders [17]. Therefore, HT holds promise as a valuable commodity within the food, pharmacological, and cosmetic industries.

The native free HT content of olive leaves is very low, less than 0.8%. Moreover, additional factors contribute to high costs of HT production, including difficulties in synthesizing HT chemically, as well as low final yields and expensive isolation of HT [18]. Meanwhile, great strides have been made using microbial enzymes to achieve bioconversion of many by-products. Enzymatic methods possess multiple advantages over conventional chemical conversion processes, such as milder reaction conditions, generation less environmental pollution, higher conversion rates, etc. Such advantages have made development of microbial enzyme bioconversion systems a main focus of research worldwide and justify development of similar systems for HT production.

Hydrolytic conversion of OLE to HT has already been achieved using enzymes of *β-glucosidase* (BG) and esterase, and the glucosidic bond was broken by BG; the ester bond was broken by esterase [19]. BG is a key enzyme that has also been used for OLE conversion to HT by facilitating the release of HT and glucose from both verbascoside and HT glucoside and also by catalyzing hydrolysis of carbohydrates and cleavage of bonds present in glycosides [20]. Several researchers have studied HT production using BG hydrolysis from olive mill waste [21], as well as from olive leaves [22, 23]. Meanwhile, hemicellulase has been used to hydrolyze OLE to HT [24]. Recently, hydrolysis by cellulase to bioconvert OLE to HT from olive leaf extract has been attempted, but has only generated low HT yield [24].

In this study, two cellulolytic enzymes with high BG activity, Novozymes CTec2 and commercial cellulase KDN (Qingdao, People's Republic of China), were examined. Olive leaf extract was enzymatically hydrolyzed by cellulase to convert OLE into HT. The contents of OLE and HT in the reaction solution were monitored during the process, and the bioconversion conditions were subsequently optimized. The goal of this study was to achieve both a high degradation rate of OLE and a high yield of HT.

2. Material and Methods

2.1. Raw materials and enzymes

An OLE extract containing 48.43% of OLE by mass was produced from olive leaves and was purchased from Sciphar (Shanxi, People's Republic of China). Cellulase Novozymes CTec2 (Celluclast, Cellic® CTec2) was supplied by Novozymes North America (Franklinton, NC, USA). A commercial cellulase preparation with a trade name of Kandien (KDN) was purchased from KDN Biotech Group (Qingdao City, People's Republic of China). Thermophilic BG, which was obtained by overexpressing the thermostable BG gene from *Thermotoga petrophila* into *Escherichia coli* [25], was kindly provided by Nanjing Forestry University. Other commercial enzyme preparations are described in Table 1.

TABLE 1 *Enzymatic hydrolysis of OLE by different enzymes*

Type of enzyme	*Source of enzyme*	*Origin of enzyme*	*Enzyme dosage (U/g OLE)*	*Temperature (°C)*	*pH*	*Degradation rate of OLE (%)*	*Yield of HT (%)*
β-glucosidase	Sigma	Almond	100	37	5	33.94 ± 1.73	6.59 ± 0.40
β-glucosidase	Novozyme 188	*Aspergillus niger*	100	50	4.8	68.41 ± 1.30	19.83 ± 0.29
β-glucosidase	Thermophillic BG (Nanjing, People's Republic of China)	Recombinant, expressed in *E. coli*	100	90	5	71.34 ± 1.36	40.34 ± 0.34
cellulase	[a]Novozymes CTec2	*Trichoderma reesei*	FPA, 5; BG, 100	50	4.8	88.42 ± 1.24	20.84 ± 0.39
Cellulase	[a]KDN (Qingdao, People's Republic of China)	*Trichoderma reesei*	FPA, 7; BG, 100	55	5	87.16 ± 0.73	20.03 ± 0.17
cellulase	Sigma	*Aspergillus niger*	100	37	5	13.74 ± 0.66	4.25 ± 0.13
cellulase	Youtell,UTA-8,(Hunan, People's Republic of China)	*Trichoderma reesei*	100	50	4.8	68.33 ± 1.66	14.19 ± 1.34
cellulase	Sukahan (Weifang, People's Republic of China)	*Trichoderma reesei*	100	55	5	20.70 ± 0.73	5.29 ± 0.17
cellulase	Sigma	*Bacillus subtilis*	100	30	7.5	46.71 ± 0.72	4.54 ± 0.22
Hemicellulase	Sukahan (Weifang, People's Republic of China)	*Trichoderma reesei*	100	55	5	19.37 ± 0.34	5.33 ± 0.40
Hemicellulase	Sigma	*Aspergillus niger*	100	40	4.5	6.62 ± 0.11	2.49 ± 0.04
Esterase	Sigma	Porcine liver	100	25	8	47.00 ± 1.25	4.72 ± 0.17
Lipase	Sigma	*Aspergillus niger*	100	40	8	70.35 ± 0.75	5.54 ± 0.42
Xylanase	Sigma	Expressed in *Aspergillus oryzae*	100	50	4.8	15.79 ± 1.12	3.50 ± 0.13

[a]Since Novozyme CTec2 and KDN with high BG activity, the dosages of the two enzyme preparations were based on BG activity.

2.2. Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade methanol was purchased from Merck (Darmstadt, Germany). Bovine serum albumin, *p*-nitrophenyl-β-D-glucopyranoside (pNPG), OLE (≥98%), and HT (≥98%) used as standards were purchased from Sigma–Aldrich (Shanghai, People's Republic of China). All chemicals used were of analytical grade.

2.3. Determination of enzyme activities and protein concentrations

Filter paper activity (FPA) was measured according to standard IUPAC (International Union of Pure and Applied Chemistry) procedures [26]. The definition of one filter paper unit (FPU) is the amount of enzyme that generates 1 μmol of glucose equivalents from 50 mg of Whatman No. 1 filter paper per minute. BG activity was determined from the amount of *p*-nitrophenol (*p*-NP) produced by enzymatic degradation of pNPG [27]. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-NP. Activity measurements of esterase [28], lipase [29], hemicellulase, and xylanase [30] were also conducted.

2.4. Analysis of OLE and HT

Analysis of OLE and HT was performed using an Agilent 1260 Infinity HPLC system. An Eclipse XDB-C18 column (250 × 4.6 mm, 5 μm) was used as the stationary phase. The mobile phase was 0.2% phosphoric acid in water versus 100% methanol. The flow rate was 0.6 mL Min^{-1}. Samples were detected by a UV–visible spectrophotometer at 230 nm. Compounds were quantified and identified by comparison of retention times and peak areas.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis experiments were conducted in 125-mL Erlenmeyer flasks with a working volume of 50 mL and a shaking speed of 150 rpm. OLE extract was used as the substrate. The amount of enzyme, temperature, and time was changed according to the goal of each experiment. After hydrolysis, the hydrolysates were centrifuged at 9,016*g* for 5 Min to remove insoluble residues.

2.6. Hydrochloric acid hydrolysis

Hydrochloric acid hydrolysis experiments were performed in 125-mL Erlenmeyer flasks with 50 mL hydrochloric acid (0.5 mol L^{-1}) and 20 g L^{-1} OLE extract. Hydrolysis was carried out at 75 °C and 150 rpm on a shaker incubator for 7 H. Then, the solutions were neutralized with sodium hydroxide to pH 6 to 7 and the samples were centrifuged at 9,016*g* to remove insoluble residues.

2.7. Calculations

The degradation rate of OLE was calculated according to the following equation:

$$R = \left(1 - \frac{W_1}{W_0}\right) \times 100$$

where R is the degradation rate of OLE (%), W_1 is the quantity of OLE after hydrolysis (g), and W_0 is the quantity of OLE before hydrolysis (g).

The percent yield of HT was calculated as the actual HT quantity obtained divided by the theoretical maximum HT quantity present in the hydrolysate (as calculated from the known initial amount of OLE in the extract):

$$Y = \frac{W_{HT}}{W_{OLE} \times 0.285} \times 100\%.$$

where Y is the yield of HT (%), W_{HT} is the quantity of HT after hydrolysis (g), W_{OLE} is the quantity of OLE before hydrolysis (g), and 0.285 is the theoretical coefficient for conversion of OLE to HT in the hydrolysate. Since the complete hydrolysis of 1 mol OLE (540 g) results in 1 mol HT (154 g), the stoichiometric value of HT derived from 1 g of OLE is 0.285 g. All experiments were carried out in duplicate to verify reproducibility of the results. Each data point presented was the average of duplicate determinations.

3. Results and Discussion

3.1. Enzyme selection for bioconversion of OLE to HT

This study aimed to use an extract of agricultural waste olive leaf to produce the high value-added product HT through bioconversion. The key goal was to obtain both a high OLE degradation rate and a high yield of HT. The glucosidic bond and ester bond in the structure of OLE (Fig. 1) are the targets for the enzymatic degradation. Various types of enzymes, which could attack the two bonds, have been widely studied in the literature. Fourteen enzyme preparations were tested in this work, and the better enzymes were chosen for further investigation.

3.1.1. The performance of various enzyme sources for OLE hydrolysis

OLE extract from *Olea europaea* L. was hydrolyzed using 14 commercial enzyme preparations at their optimal temperatures and pH values for 24 H. A high quantity of each enzyme was used, 100 unit per gram of OLE extract, to rule out insufficient enzyme activity as a cause of low yields. Since Novozymes CTec2 and KDN cellulase possess high BG activity, the dosages of the two enzyme preparations were based on 100 U of BG activity. The substrate concentration was 20 g L^{-1}.

As shown in Table 1, various BG preparations possessed relatively high abilities to degrade OLE. Both Novozymes 188 and thermophilic BG could hydrolyze about 70% of OLE in 24 H. The yield of HT, however, was lower than expected and was 40.34% for thermophilic BG and 19.83% for Novozymes 188. Cellulases performed differently in 24 H; Novozymes CTec2, KDN, and Youtell UTA-8 degraded 88.42%, 87.16%, and 68.33% of OLE, respectively, although the yield of HT was much lower than that obtained by enzymatic hydrolysis with thermophilic BG. Other cellulases tested showed very low abilities to degrade OLE and form HT. Lipase could hydrolyze 70.35% OLE in 24 H, but the yield of HT was negligible.

Hydrolyses using other enzyme preparations, including two hemicellulases, one esterase and one xylanase, exhibited low OLE degradation rates and low HT yields.

The chemical structure of OLE contains an ester bond and a glucosidic bond, each of which can be cleaved by hydrolases. Hydrolysis of the ester bond produces HT, whereas cleavage of the glucosidic bond releases glucose. Several researchers have studied enzymatic hydrolysis to convert OLE to HT using BG from various sources with variable results. Khoufi et al. [21] studied the enzymatic hydrolysis of olive wastewater to produce HT using BG from *Aspergillus niger* broth culture, showing an HT concentration increase from 0.05 to 0.80 g L^{-1}. Hamza and Sayadi [22] reported, using an initial concentration 8.3 g L^{-1} of OLE in olive leaf extract, that OLE hydrolysis using *A. niger* BG achieved an HT concentration of 0.55 g L^{-1}. Jemai et al. [31] hydrolyzed olive leaf extract (4.32 g L^{-1} of OLE) using BG from almonds at 37 °C and pH 7 to produce a small amount of HT and 3.82 g L^{-1} of oleuropein aglycon. Results using recombinant BG from hyperthermophilic archaeon *Sulfolobus solfataricus* were also reported [32]. In several studies, using hemicellulase from *A. niger*, generally low catalytic efficiency has been reported [33]; however, in one study a very high OLE degradation rate of 98.54% was reported [24]. Meanwhile, a high OLE degradation rate of 90% has also been reported using microbial conversion with *Lactobacillus plantarum* 6907 at 30 °C for 10 days [34]. But the yields of HT in these studies were all low.

In the present work, 14 enzyme preparations were screened. Cellulase CTec2, KDN, and thermophilic BG showed relatively high abilities to degrade OLE and produce high HT yields. These enzyme preparations were used for further study.

3.1.2. Enzymatic hydrolysis of OLE by cellulase CTec2 and thermophilic BG

For the remainder of this study, the two selected enzymes, cellulase CTec2 and thermophilic BG, were employed to study hydrolysis of OLE to HT. A substrate concentration of 20 g L^{-1} was used. The activity of CTec2 was 5 FPU per gram of OLE extract (BG activity was equal to 100 U per gram of OLE) and that of thermophilic BG was 100 U per gram of OLE extract. The optimal recommended temperature was 50 °C for CTec2 and 90 °C for thermophilic BG. Enzymatic hydrolysis was carried out at pH 4.8 using a longer reaction time (72 H) than previously used. Since the two enzymes react at different temperatures, to investigate if a combination of enzymes might result in synergistic effects, four sets of experiments were designed: (1) CTec2 at 50 °C, (2) thermophilic BG at 90 °C (the control), (3) CTec2 at 50 °C for 12 H followed by raising temperature to 90 °C without the addition of thermophilic BG, and (4) CTec2 at 50 °C for 12 H followed by addition of thermophilic BG at 90 °C.

As illustrated in Fig. 2, extension of reaction time improved the degradation of OLE and the formation of HT. Hydrolysis with CTec2 at 50 °C alone degraded 75.50% of OLE and formed 25.36% of HT in 12 H. After 24 H, the degradation rate of OLE and the yield of HT increased to 89.01% and 33.36%, respectively. At 48 H, the degradation rate of OLE reached

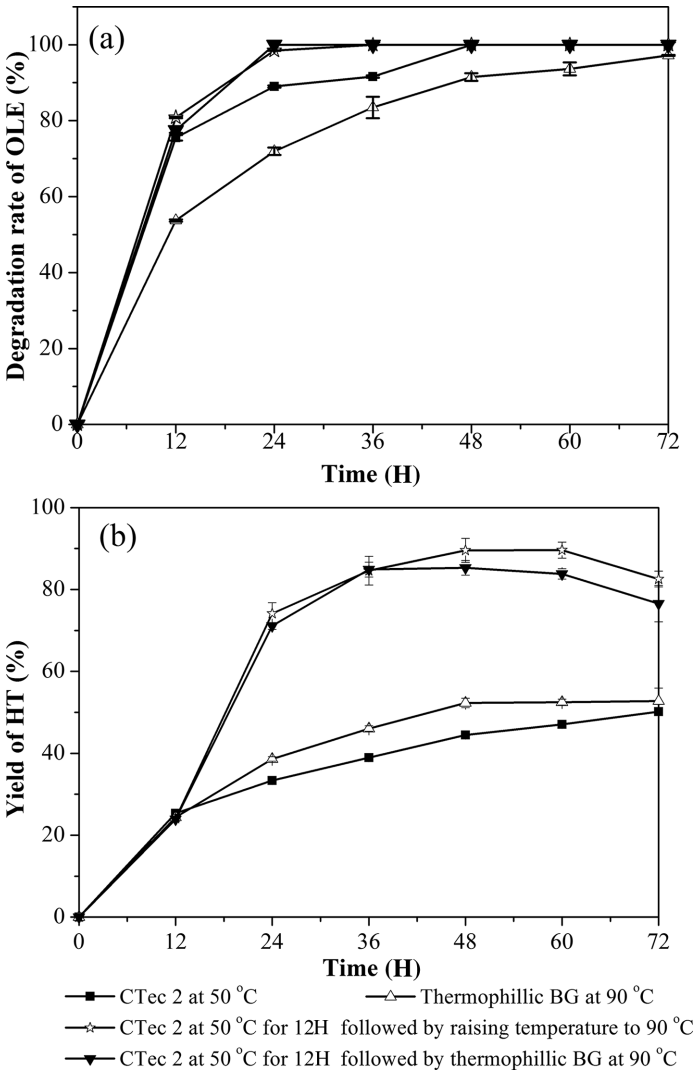

FIG. 2 *Profile of enzymatic hydrolysis of OLE (a) to produce HT (b).*

100% with a HT yield of 44.46%. Thermophilic BG at 90 °C exhibited similar trends, but overall its degradation ability was lower than that of CTec2; the OLE degradation rate was 53.71% in 12 H, 71.92% in 24 H, and 91.47% in 48 H. However, after 12 H, the HT yields were higher for all time points than were observed for CTec2 hydrolysis. After 48 H of hydrolysis, a yield of 52.31% HT was achieved for thermophilic BG.

Notably, a significant improvement in both OLE degradation and HT formation was observed after hydrolysis by CTec2 at 50 °C for 12 H followed by a temperature increase to 90 °C without the addition of thermophilic BG. At 24 H, the OLE was almost exhausted, whereas the HT yield jumped to 74.14%, a value much higher than the 20.84% yield obtained for CTec2 hydrolysis performed at a constant 50 °C (Table 1). Moreover, the HT yield remained consistently high for the remainder of

the high-temperature hydrolysis reactions: 84.62% at 36 H, 89.57% at 48 H, and 89.63% at 60 H.

For hydrolysis using CTec2 followed by addition of thermophilic BG at 90 °C, similar trends in OLE degradation and HT formation were observed, whereby the yield of HT decreased gradually after reaching its peak value, from 85.89% at 36 H followed by 85.30% at 48 H, 83.81% at 60 H, and 76.53% at 72 H. These results indicate that CTec2 and high temperature played an important role in the hydrolysis of OLE, regardless of added thermophilic BG. The likely explanation for this observation is that CTec2 cleaved the glycosidic bonds and loosened the molecular structure of OLE at 50 °C, whereas the release of HT occurred at 90 °C even though CTec2 was deactivated at this temperature. The addition of thermophilic BG to the CTec2 hydrolysis reaction did not improve OLE degradation and HT formation, probably because CTec2 had already catalyzed conversion via its high BG activity.

Moreover, the addition of thermophilic BG achieved even higher BG activity that actually promoted the transglycosylation activity of BG, which led to the aggregation of small molecules and ultimately decreasing HT yield (Fig. 2b).

Cellulase is a complex enzyme system containing endo-1,4-β-glucanase (EC 3.2.1.4), exo-1, 4-β-D-glucanase (EC 3.2.1.74) and BG (EC 3.2.1.21). These enzymes exhibit synergistic effects during hydrolysis [35]. In the enzymatic hydrolysis at 50 °C, glucosidic bonds are easily cleaved by cellulase, whereas the ester bonds are not cleaved extensively. Therefore, the degradation rate of OLE could reach 80.86%, but the yield of HT could only reach 24.09%. When the temperature was then increased to 90 °C, the ester bonds were likely easily broken, resulting in a high HT yield. Therefore, enzymatic hydrolysis by cellulase and chemical degradation by high temperature are both required for efficient bioconversion of OLE to HT.

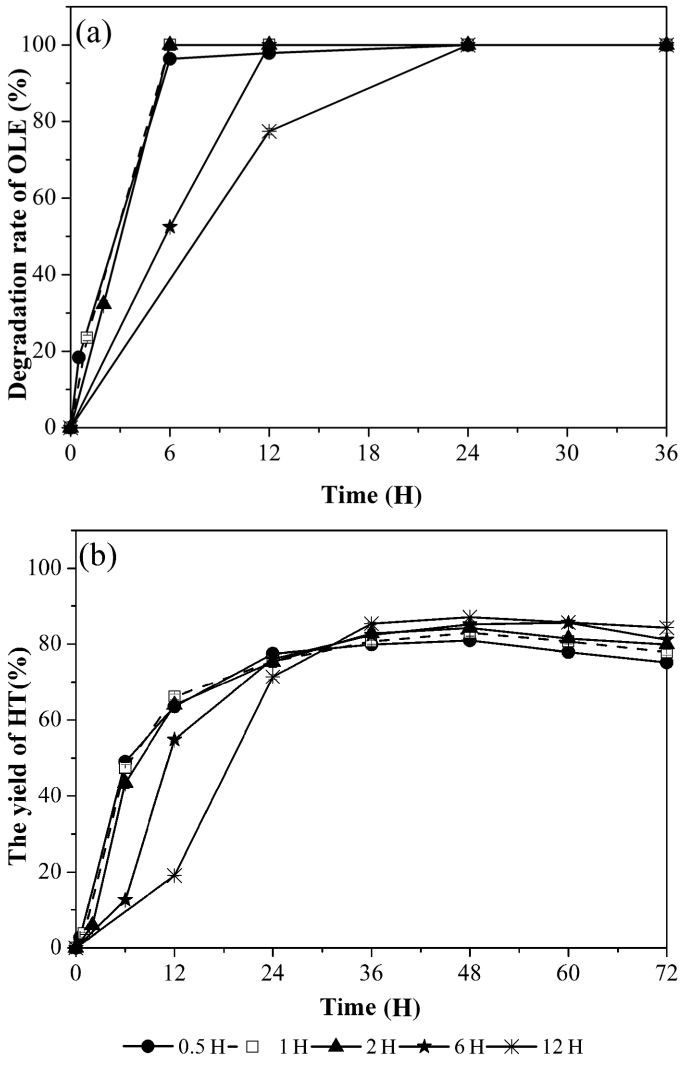

FIG. 3 *Effect of reaction time at 50 °C on enzyme hydrolysis of OLE (a) to produce HT (b).*

3.2. Investigation of hydrolysis optimal conditions

Because the addition of thermophilic BG did not improve OLE degradation and HT formation, in subsequent experiments only cellulase was used.

3.2.1. Effect of reaction time at 50 °C on enzymatic hydrolysis

The optimal reaction temperature for cellulase CTec2 is 50 °C. Enzymatic hydrolysis was conducted at this temperature for 0.5H, 1, 2, 6, and 12 H (the control), and then the temperature was raised to 90 °C. The concentration of OLE extract was 20 g L^{-1}, and the activity of CTec2 used was 5 FPU per gram of OLE extract. Figure 3 shows that various reaction times resulted in varying OLE degradation rates and HT yields; with increasing reaction time, these values gradually became similar to one another. For example, when enzymatic hydrolysis was conducted at 50 °C for 0.5 H, the degradation rate of OLE was only 18.37% and the yield of HT was very low (2.68%). These values were much lower than those for the reaction carried out at 50 °C for 12 H, which were 77.40% for OLE degradation and 19.07% for HT formation. However, after the temperature was increased to 90 °C, at 24 H OLE was nearly exhausted and the yield of HT exceeded 70% in all cases. At 36 H, the HT yields of hydrolysis at 50 °C for 0.5, 1, 2, 6, and 12 H were 79.89%, 80.71%, 82.82%, 82.44%, and 85.40%, respectively. A longer reaction time resulted in slightly better yields. However, such small increases obtained by raising the temperature to 90 °C for a longer period of time required additional energy for heating and would thus increase costs. Therefore, for acceptable yields at a lower cost, a reaction time of 12 H was selected.

3.2.2. Effect of high temperature on hydrolysis

To determine the best high temperature for the formation of HT, enzymatic hydrolysis was first performed at 50 °C for 12 H then the temperature was increased to 70, 80, 85, or 90 °C (the control) for 24 H. The initial OLE concentration of the extract was 20 g L^{-1}, and the initial enzyme activity of CTec2 was 5 FPU per gram of OLE extract.

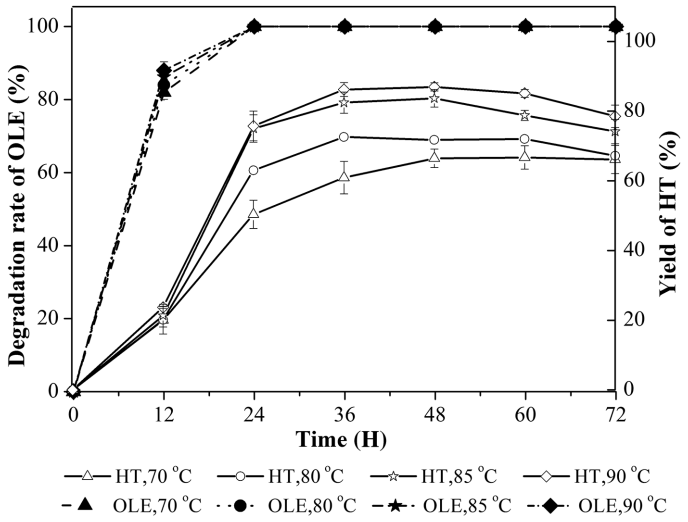

FIG. 4 *Effect of high temperature on the degradation of OLE and the formation of HT.*

As illustrated in Fig. 4, the degradation of OLE was successful, as expected. After 24 H of hydrolysis, OLE was consumed completely for all of the temperatures tested. The formation of HT, however, exhibited notable differences. After conducting hydrolysis 36 H, hydrolysis with high temperatures at 70, 80, 85, and 90 °C resulted in HT yields of 60.92%, 72.59%, 82.44%, and 86.19%, respectively. Extending the reaction time did not change the overall trend, which could be explained by the fact that cleavage of the ester bond requires a high temperature. This observation is supported by research of Fergusson et al. [36], which reported high-temperature ester hydrolysis could be used to recover alcohol from one or more fatty acid alcohol esters or one or more diacid alcohol esters. Briante et al. [37] also demonstrated high-temperature enzymatic hydrolysis of OLE, but used recombinant BG from *hyperthermophilic archaeon* immobilized onto a chitosan matrix.

3.2.3. Effect of substrate concentration on enzymatic hydrolysis

The substrate concentration is one of the main factors affecting catalytic efficiency. Therefore, the effects of different substrate concentrations were studied. Enzymatic hydrolysis was first performed at 50 °C for 12 H then the temperature was increased to 90 °C for the following 36 H. The initial enzyme activity of CTec2 was 5 FPU per gram of OLE extract. Substrate concentrations of 14, 17, 20, 25, and 33 g L^{-1} were studied. Table 2 shows the results of OLE's degradation and HT's formation after 48 H of enzymatic hydrolysis. When the substrate concentration was below 25 g L^{-1}, the degradation rates of OLE were all 100% and the yields of HT were similar to one another at approximately 84%. However, in the case of 33 g L^{-1} of substrate, the HT yield was about 15% less than that of the above tests, even though the degradation rate of OLE was still very high. The results may be explained by substrate

TABLE 2 *Effect of substrate concentration on enzymatic hydrolysis*

Substrate concentration (g L^{-1})	Degradation rate of OLE (%)	Yield of HT (%)
14	100	83.94 ± 1.09
17	100	83.79 ± 1.30
20	100	84.96 ± 1.75
25	99.37 ± 0.67	83.89 ± 0.75
33	99.68 ± 0.12	71.58 ± 2.29

accessibility to enzyme. When substrate level was relatively low, increases in the substrate concentration led to an increase in the reaction rate. Within a moderate substrate concentration range, higher substrate concentration provided more opportunities for enzyme and substrate to interact, promoting more hydrolysis. However, when the substrate concentration was too high, the enzyme solution became relatively more crowded with both substrate and reaction products. This crowding decreased diffusion efficiency, ultimately inhibiting enzyme-driven hydrolysis and reducing the hydrolysis rate. Therefore, based on the results presented in Table 2, a moderate substrate concentration of 20 g L^{-1} was ultimately chosen for further study.

3.2.4. Effect of enzyme activity level on enzymatic hydrolysis

Because overall costs of processing operations decrease profitability, reducing the cost of cellulase used in hydrolysis is a key consideration in designing an industrial process. Therefore, the use of an appropriate amount of enzyme is of particular importance in the present study. In this paper, the effect of number of enzyme activity units on hydrolysis was investigated. The CTec2 units of activity added to reactions were 1, 2, 5, 10, 20, and 30 FPU per gram of OLE extract. For all samples, enzymatic hydrolysis was initiated at 50 °C for 12 H, then the temperature was raised to 90 °C. A substrate concentration of 20 g L^{-1} was adopted for all tests.

As shown in Fig. 5, if the enzyme amount was low (1 and 2 FPU per gram of OLE extract in this work), the degradation rates of OLE and the HT yields of HT were lower and were more similar to one another than at higher enzyme activities. When enzyme activity was above 5 FPU per gram of OLE extract, cellulase activity did not significantly affect the OLE degradation rate and HT yield. For instance, at 48 H hydrolysis of samples containing enzyme activities of 5, 10, 20, and 30 FPU per gram of OLE extract resulted in complete consumption of OLE, with resulting HT yields of 86.25%, 89.06%, 87.95%, and 84.51%, respectively. It is believed that the adsorption of cellulase to substrate has a certain saturation point. Below this saturation point, increasing amounts of enzyme activity caused

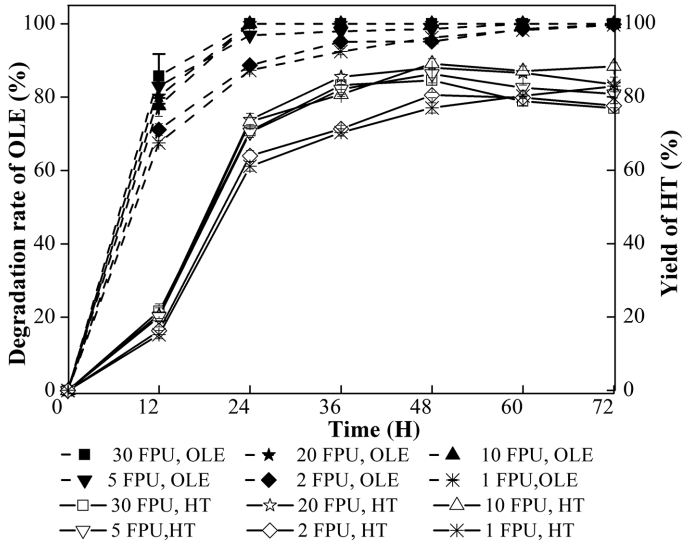

FIG. 5 *Effect of enzyme dosage on the degradation of OLE and the formation of HT.*

the hydrolysis rate to increase, since enzyme was the limiting factor. However, when the enzyme activity in a reaction became excessive, a substrate was the limiting factor and therefore no continuous increase of the reaction rate could be detected with increases in enzyme activity. Because enzyme costs must be considered in calculating overall costs, use of the lowest necessary amount of enzyme would be beneficial. Thus, in this work the optimal enzyme activity level was selected as 5 FPU per gram of OLE extract.

3.3. Time course of hydrolysis under optimal conditions

The time course of enzymatic hydrolysis of OLE to produce HT using the optimal conditions described above was examined. The concentration of OLE extract was 20 g L^{-1}, and the optimal enzyme activity level of cellulase CTec2 and KDN was 5 FPU per gram of OLE extract. Enzymatic hydrolysis was conducted at pH 4.8 and 50 °C for 12 H, then the temperature was raised to 90 °C. After various reaction durations, the OLE degradation rates and the HT yields were determined. The results are presented in Table 3.

The degradation of OLE by CTec2 was achieved in the 50 °C stage, where the OLE concentration decreased from an initial of 9.68 to 2.02 g L^{-1} in 12 H, with an OLE degradation rate of 79.58%. At 24 H, no OLE was detected; it is reasonable to assume that OLE was exhausted before this time point. Notably, the formation of HT was not synchronous with the degradation of OLE. The yield of HT was only 24.83% in 12 H and 73.32% in 24 H. After hydrolysis for 36 H, the yield of HT reached 84.03%. The highest yield, 88.90%, was detected at 48 H after which the yield of HT decreased slowly, possibly due to further transformation of HT into other phenolic compounds under high-temperature conditions. Cellulase KDN performed similarly to CTec2.

Bioconversion of OLE into HT using other enzymes has been studied by several research groups. When an olive leaf extract with a high OLE concentration of 81.04% was used in a hydrolysis reaction with hemicellulase, a high OLE degradation rate of 98.54% was observed with a low yield of HT [24]. When BG from *A. niger* was used for OLE hydrolysis by Hamza and Sayadi [22], a yield of HT was also low. These studies suggest that enzymatic hydrolysis with hemicellulase or BG could cleave the glucoside bond present in the molecule of OLE, but neither enzyme could break the ester linkage completely.

The OLE molecule is composed of three structural units: a polyphenol (HT), elenolic acid, and a glucose molecule. In the bioconversion process, OLE is first hydrolyzed by an enzyme to form oleuropein aglycon with the release of a molecule of glucose. The oleuropein aglycon easily undergoes rearrangement and is then converted into HT and elenolic acid by chemical hydrolysis [37]. Enzymatic hydrolysis, regardless of the enzyme employed, may facilitate release of glucose from OLE to promote formation of oleuropein aglycon, resulting in a high OLE degradation rate. However, oleuropein aglycon might be difficult to convert into HT and elenolic acid under the mild conditions of enzymatic hydrolysis. In this study, a similar phenomenon might be occurring; after enzymatic hydrolysis at 50 °C for 12 H, the yield of HT was still very low, even though the OLE had been degraded extensively. A high yield of HT was only obtained when the temperature was raised to 90 °C, presumably because chemical hydrolysis was promoted at high temperature to achieve cleavage of ester bonds. Therefore, the high HT yield of 88.90% in the present work may be attributed to both enzymatic hydrolysis at 50 °C and chemical hydrolysis at 90 °C.

Although BG is efficient for the deglycosylation of OLE, the formation of HT, however, depends on the synergism among varied enzymes to convert oleuropein aglycon into HT and elenoic acid. In this study, two cellulase preparations, CTec2 and KDN, performed very well both in OLE degradation and HT formation as shown in Table 3. CTec2 enzyme preparation is an optimized enzyme complex consisting of endoglucanases, cellobiohydrolases, and BG, whereas KDN cellulase is a commercial enzyme product widely used in feed, food, paper, and textile industries. The high BG activity and rich enzyme components of the two enzyme preparations might lead to the successful bioconversion of OLE into HT, although the mechanism requires further investigation.

3.4. HPLC analysis of HT and OLE during hydrolysis

Analyses of phenolic compounds were monitored by HPLC. The HPLC chromatograms of enzymatic hydrolysis of OLE by CTec2 at 0, 12, and 48 H are shown in Fig. 6. For comparison, the results of OLE hydrolysis by dilute HCl were also included. The retention times of HT and OLE were 6.029 and 18.513 Min, respectively.

TABLE 3 *The course of hydrolysis of OLE to HT by two cellulase preparations*

	Novozymes CTec2				*KDN*			
Time (H)	*Concentration of OLE (g L^{-1})*	*Degradation rate of OLE (%)*	*Concentration of HT (g L^{-1})*	*Yield of HT (%)*	*Concentration of OLE (g L^{-1})*	*Degradation rate of OLE (%)*	*Concentration of HT (g L^{-1})*	*Yield of HT (%)*
0	9.68 ± 1.23	ND	ND	ND	9.60 ± 0.57	ND	ND	ND
12	2.02 ± 0.89	79.58 ± 0.29	0.70 ± 0.69	24.83 ± 0.50	1.98 ± 1.21	78.32 ± 0.45	0.68 ± 0.89	23.55 ± 0.39
24	ND	100	2.11 ± 2.14	73.32 ± 1.95	ND	100	2.10 ± 1.02	72.55 ± 0.58
36	ND	100	2.47 ± 1.32	84.03 ± 1.36	ND	100	2.42 ± 0.48	83.91 ± 0.21
48	ND	100	2.67 ± 0.56	88.90 ± 1.87	ND	100	2.61 ± 0.69	87.64 ± 0.16
60	ND	100	2.70 ± 0.48	87.95 ± 0.66	ND	100	2.69 ± 0.24	87.05 ± 1.01
72	ND	100	2.72 ± 1.78	86.63 ± 0.82	ND	100	2.71 ± 0.37	86.24 ± 0.57

ND: Not detected.

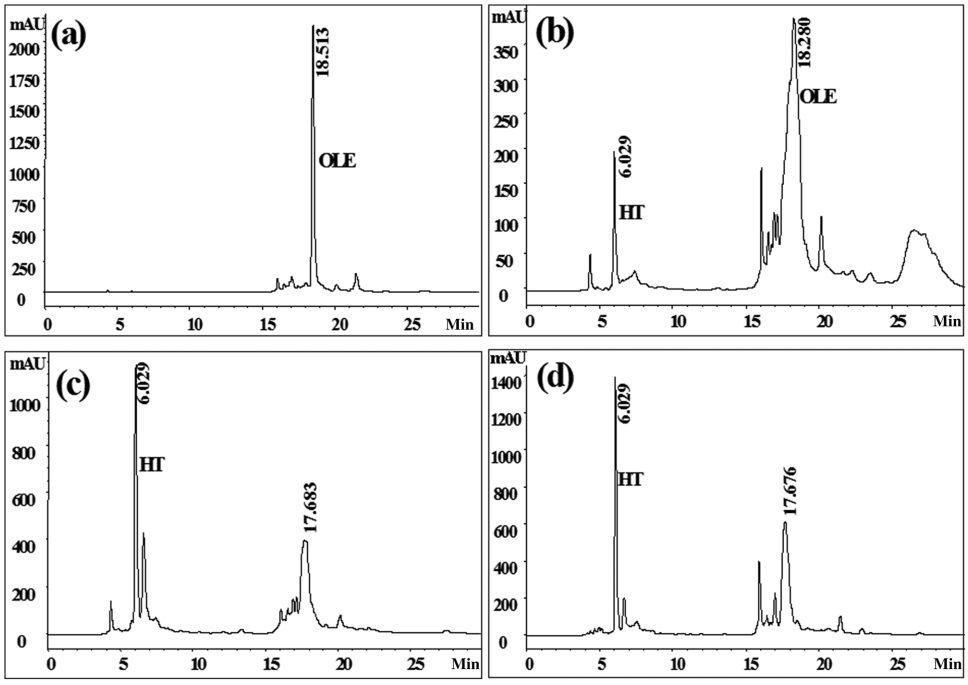

FIG. 6 *HPLC chromatography of OLE and HT during enzymatic hydrolysis by CTec2 for 0 H (a), 12 H (b), and 48 H (c), compared with the result of HCl hydrolysis (d).*

As shown in Fig. 6a, the OLE extract was rich in OLE and contained low quantities of other phenolic compounds. After enzymatic hydrolysis for 12 H (Fig. 6b), the OLE peak substantially decreased, the HT peak was easily detected, and a number of wide peaks in the range of 16–18 and 24–30 Min were observed. These wide peaks may represent the intermediate products from OLE degradation. When enzymatic hydrolysis was conducted for 48 H (Fig. 6c), the OLE peak was hardly detectable whereas the HT peak was significantly increased. Moreover, the intermediate products at 24–30 Min basically disappeared whereas the unidentified products appearing at 16–18 Min were still present at high density. Notably, this 48 H result strongly resembles the peak profile of HCl hydrolysis (Fig. 6d).

The hydrolysis of OLE by HCl resulted in an OLE degradation rate of 97.73% and HT yield of 94.07% (data not shown). These results are comparable to results reported here for

enzymatic hydrolysis followed by high-temperature hydrolysis, where an OLE degradation rate of 100% and HT yield of 88.90% were obtained. HCl hydrolysis has advantages of shorter reaction time, higher HT yield, and a proven track record as a valuable processing method. However, HCl is a corrosive acid and thus exerts harsh effects on processing equipment. Moreover, even after neutralization with base, the separation of HT from the solution is difficult and costly. On the other hand, enzymatic hydrolysis as described here may be conducted under mild conditions with creation of little environmental pollution and may eventually replace hydrolysis by HCl for industrial-scale HT production.

4. Conclusions

Enzymatic hydrolysis using two cellulase preparations with high BG activity, Novozymes CTec2 and commercial cellulase KDN, followed by high-temperature hydrolysis was conducted to produce the high value-added product HT from OLE presented in olive leaf extract. In this work, the optimal conditions of hydrolysis were investigated. Enzymatic hydrolysis was carried out at 50 °C for 12 H and then the temperature was increased to 90 °C. After 48 H of hydrolysis, the degradation rate of OLE was 100% and the yield of HT reached 86–88%. High yield of HT requires both enzymatic hydrolysis at 50 °C and chemical hydrolysis at 90 °C. The former stage releases glucose from OLE, whereas the latter stage promotes the cleavage of the ester bond to form HT.

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6. Disclosure

The authors have approved the final article and declare no conflict of interests.

7. References

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