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Immobilization of beta-glucosidase purified from mandarin (*Citrus reticulata*) fruit to superparamagnetic nanoparticles and its aroma quality enhancing effect

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Abstract

This paper reports on novel and efficient enhancement effects of fruit juice aroma using immobilized β-glucosidase, the enzyme involved in important functions in living organisms, onto superparamagnetic nanoparticles Fe_3O_4 via carbodiimide β-glucosidase was purified from mandarin (*Citrus reticulata*) using ammonium sulfate precipitation and hydrophobic interaction chromatography. To be used in this study, superparamagnetic nanoparticles were synthesized and then the shape, size, and magnetism properties of the nanoparticles were characterized. The purified enzyme was immobilized on the nanoparticles. The optimum temperature for β-glucosidase (40 °C) was increased by 10 °C after immobilization, while the optimum pH values of free and immobilized β-glucosidase were 5.5. While the K_m and V_{max} values of the free enzyme were 0.264 mM and 294 EU, immobilized enzyme's K_m and V_{max} were 0.222 mM and 370 EU, respectively. In addition, it was determined that the storage stability of the immobilized enzyme was higher than the free enzyme. When the effect of some metal ions on the enzyme activity was examined, it was observed that Fe^{+2} increased the enzyme activity while other metals inhibited it. According to the results obtained, the immobilized enzyme had a flavor-enhancing effect on mandarin juice.

Introduction

β-Glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21) are present in both prokaryotes and eukaryotes as responsible enzymes for selectively catalyzing the hydrolysis of β-glycosidic bonds either between two glycone molecules or between glycone and aglycone residues. They play various essential key roles in physiological activities and biotechnological processes, including food quality and flavor enhancement (Turan 2008). It has been reported that, following the hydrolysis of glycosidic bond by using beta-glucosidase activity, the release of potential aroma constituents increases the aromatic quality of whole fruit or processed fruit products and beverages (Fan *et al.* 2011). However, glucosidase has been reported to show mostly lower activities during food processes, and therefore exogenous glucosidase is proposed to be used for enhancing the aroma quality of fruits and their products (Su *et al.* 2010). Since the purified enzymes are essential for exogenous uses, the purification processes of the enzymes require specific techniques that result in high costs.

Removal of the free enzyme without activity loss is mostly impossible following catalysis in these types of industrial applications. Addition of the inhibitors for the termination of the catalytic reactions causes extra contaminations of the processed foods. Separation and removal of contaminants from processed food products require additional and more complicated techniques. In addition, the aroma quality of the fruit beverages may be remarkably altered by the loss of volatile aromas during long industrial processes. Considering these kinds of unfavorable outcomes, immobilized enzyme systems have been established and primarily preferred for aroma enhancement applications in recent years. The use of immobilized beta-glucosidases from different sources for these applications has also been reported. Shoseyov *et al.* (1990) used *Aspergillus niger* endo-beta-glucosidase for immobilization on acrylic beads and corn stover cellulose particles to enrich the flavor of wine and passion fruit juices. Gueguen *et al.* (1997) showed enhancement of the aromatic quality of Muscat wine using of *Candida molischiana* 35M5N beta-glucosidase immobilized to Duolite A-568 resin. Gallifuoco *et al.* (1999) immobilized beta-glucosidase in chitosan pellets to study the winemaking industry. Yan *et al.* (2010) immobilized and determined the activity of beta-glucosidase on different soil colloids. Fan *et al.* (2011) reported the immobilization of orange beta-glucosidase by three different methods and used it to release the bound volatile compounds in orange juice. On account of the fact that, the tea plant glucosidases show low activity under natural conditions and are destroyed to a high degree during the tea manufacturing processes of withering, rolling, and fermentation. Su *et al.* (2010) preferred to immobilize a commercially purchased beta-glucosidase on alginate by the crosslinking–entrapment–crosslinking method and showed its aroma-increasing effect on tea beverages. Pombo *et al.* (2011) isolated an extracellular β-glucosidase from *Issatchenkia terricola* and immobilized it onto Eupergit C for aroma enhancement of white Muscat wine.

Magnetic nanoparticles consist of magnetic elements such as iron, cobalt, nickel, and their alloys (Liu *et al.* 2020). Magnetic nanoparticles not only have special magnetic properties such as superparamagnetism but also have unique physical properties, biocompatibility, stability (Shabestari Khiabani *et al.* 2017). Fe_3O_4 nanoparticles are widely used in separation technology (Samanta and Ravoo 2014), protein immobilization (Xu *et al.* 2009), catalysis (Liu *et al.* 2010), medical science (Lee and Kang 2017), and the environment (Guo *et al* 2015).

The most important advantage of using magnetic nanoparticles is that once the enzyme is added to the reaction mixture, it is easily removed from the environment and can be used repeatedly in this way. Immobilized enzyme can be selectively separated from a reaction mixture by the application of a magnetic field produced by a permanent magnet (Kockar *et al.* 2010).

This is the first time that this study presents the isolation and characterization of the beta-glucosidase from mandarin (*Citrus reticulata*) fruit to study the optimization of immobilization conditions to superparamagnetic iron oxide (Fe_3O_4) nanoparticles, the characterization of immobilized enzyme, and its application to the release of bound aroma constituents in mandarin to determine whether this enzyme and the method performed can be utilized in industry.

Experimental

Chemicals

Mandarin (*Citrus reticulata*) fruits used in this study were harvested in October from a field near Balikesir, Turkey. The materials, including *p*-NPG, *o*-NPG and carbodiimide, were obtained from Sigma Chem. Co. Iron (II) chloride tetrahydrate (Merck, ≥99 %), iron (III) chloride hexahydrate (Sigma-Aldrich, ≥99 %) and ammonium hydroxide (Merck, 25 % ammonium in water) were used for the synthesis. $HClO_4$ (Merck, 60 %) was used to prepare the dispersion. All chemicals were of analytical grade and used without further purification. All other chemicals were of the best available grade. Enzyme assays were measured with the aid of a Thermo Scientific Multiscango UV-Visible Spectrophotometer. The purification gel, which is used in this study, was synthesized at Balikesir University, Department of Biology laboratory (Sinan *et al.* 2006).

Preparation of magnetic nanoparticles

The iron oxide nanoparticles (IONs, Fe_3O_4) were synthesized by co-precipitation in an N_2 atmosphere at room temperature. The synthesis of IONs was based on Massart's method (Massart 1981) 1 M $FeCl_3 \cdot 6H_2O$ was dissolved in 40 mL deionized water, and 2 M $FeCl_2 \cdot 4H_2O$ was dissolved in 10 mL 2 M HCl solution. NH_4OH was added to the solution under vigorous stirring for 30 min in an N_2 atmosphere. After the reaction, the precipitate was washed three times with deionized water and dried in an oven overnight to remove the water. Dispersion of the nanoparticles was obtained by using $HClO_4$ as in the method of Massart (Massart 1981).

Characterizations

The structural characterization of the IONs was done by Phillips Analytical X-ray diffractometer using CuKα radiation (λ=1.54056 Å) between 20o<2θ<80o. Fourier transform infrared spectroscopy (FTIR, Perkin Elmer-1600 Series) was also employed to investigate the structure of IONs and the immobilization of β-glucosidase to IONs. The shape and particle size of the nanoparticles were determined by TECNAI G2 F30 model high-resolution transmission electron microscope (TEM) operating at an accelerating voltage of 200 kV. The particle size of the nanoparticles (dTEM) was measured from the image using the ImageJ program. The magnetic properties of IONs and β-glucosidase bound IONs were measured by a vibrating sample magnetometer (VSM, ADE EV9 Model) in a field range ± 20 kOe (1 Oe intervals) at room temperature.

*Purification of β-glucosidase from Mandarin (*Citrus reticulata*) by hydrophobic interaction chromatography*

In this study, the Kara *et al.* (2011) procedures were used for the purification of β-glucosidase. Firstly, mandarin fruits were washed with distilled water three times. Secondly, to prepare the crude extract, 200 g of sample tissue was cut quickly into thin slices and homogenized in a warring blender for 2 min using 100 mL of 0.1 M tris buffer (pH 8.0) including 1 M NaCl, 0.02 % (w/v) NaN_3. After filtration of the homogenate through muslin, the filtrate was centrifuged at 15,000 g for 30 min, and the supernatant was collected. The enzyme solution was treated with solid ammonium sulfate to obtain the 40 – 80 % saturation fraction by centrifuging at 15,000 rpm for 30 min. The enzyme solution was applied to the hydrophobic column (1.0 cm diameter × 10.0 cm length), pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.8) including 1.5 M $(NH_4)_2SO_4$. The enzyme was eluted using a linear gradient of 1.5 – 0.0 M $(NH_4)_2SO_4$ in the same buffer at a flow rate of 25 $mL.h^{-1}$; 1.5 mL fractions were collected. The proteins containing the highest β-glucosidase activity were combined and used as immobilization studies after confirming homogeneity by gel electrophoresis.

SDS and native polyacrylamide gel electrophoresis

SDS and Native Polyacrylamide gel slab electrophoresis of purified enzyme was carried out according to the method of Laemmli (1970). Both electrophoresis were performed in a Minigel system (Bio-Rad Laboratories, USA) using 3 % stacking and 10 % separation gels. Gels were fixed, stained with Coomassie Brilliant Blue R-250 (Sigma), and destained using standard methods to detect protein bands. The Thermo Scientific Unstained Protein Weight Marker was used to determine the size of the protein bands after electrophoresis.

Binding of β-glucosidase to superparamagnetic nanoparticles

Firstly, 0.1 g of IONs were added to 2 mL of buffer A (0.003 M phosphate, pH 6, 0.1 M NaCl) for each sample and blank tube. For the dispersion of IONs, the mixture was incubated in a sonicator for 30 min. 0.5 mL of carbodiimide was added over this mixture. The mixture was allowed to stand in the sonicator for 15 minutes. Thus, activating the surface of the nanoparticles carbodiimide was brought to the β-glucosidase enzyme, which was ready to connect. Finally, 2 mL of β-glucosidase solution (0.5 – 15 $mg.mL^{-1}$ in buffer A) was added, and the reaction mixture was sonicated for 30 min. The supernatant was used for the protein analysis.

The precipitates were washed with buffer A, then buffer B (0.1 M Tris, pH 8.0, 0.1 M NaCl), and then directly used for the measurements of activity and stability.

Free and immobilized β-glucosidase enzyme assay

During enzyme extraction and purification, free β-glucosidase activity was determined using *para*-nitrophenyl β-d-glucopyranosides (*p*-NPG) as substrate. 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 microliter plate in duplicate. After incubation at 37 ℃ for 30 min, the reaction was stopped by adding 70 µL of 0.5 M Na_2CO_3, and the color of the occurred *p*-nitrophenol formation was measured at 410 nm. Enzyme activity was expressed as µmol *p*-nitrophenol formed per minute in the reaction mixture under these assay conditions.

Immobilized β-glucosidase enzyme assay was carried out with reference to the free enzyme activity. For this purpose, 1 mL of 2.5 mM *p*-NPG substrate was added to tubes containing 0.1 g of β-glucosidase bound IONs and IONs. IONs were used as a blank. The mixture was incubated at 37 ℃ for 30 min with shaking (210 rpm). At the end of 30 min, nanoparticles were removed by using a magnet. Each 140 µL sample and blank reaction mixture were taken, and the reaction was stopped by adding 70 µL of 0.5 M Na_2CO_3, and the color that developed because of *p*-nitrophenol liberation was measured at 410 nm. Free and immobilized enzyme units were calculated from the *p*-NPG graph equation.

Stability measurement

Free β-glucosidase and immobilized β-glucosidase storage stability were measured by assaying their relative activities in buffer B at 37 ℃ after being incubated in buffer B at 4 ℃ and 25 ℃ for a required period. The stabilities of immobilized β-glucosidase and free β-glucosidase were investigated by measuring their relative activities up to 30 days. Initial activity was assumed to be 100 and the next activity results are proportioned accordingly.

Determination of kinetic and biochemical parameters

The kinetic parameters of the free and immobilized β-glucosidase enzyme using *p*-NPG as a substrate were calculated by measuring their activity in buffer B with seven different substrate concentrations at pH 5.5 and 25 ℃. The concentrations of *p*-NPG in the reaction mixture were 0.35 – 2.5 mM. A double reciprocal Lineweaver–Burk plot was used to calculate the parameters.

The biochemical parameters, pH and temperature, of free and immobilized β-glucosidase were measured. The effect of varying the pH on enzyme activity was examined using 25 mM sodium acetate (3.0 – 5.8), citrate-phosphate (3.0 – 7.0) and phosphate (6.0 – 11.0) buffers. For optimum temperature determination, the enzyme and substrate *p*-NPG solution mixtures were assayed in the range 20 – 60 ℃ for 30 min.

To study the effect of various metals on mandarin β-glucosidase activity, enzyme activity was assayed in 1 mM final concentration of Fe, Cu, Ni, Pb, Cd, Zn, Cr, Mn, Mg, and Ag. The activity results of the enzyme solution, which does not contain metal ions, were statistically compared (ANOVA) with the activity results of the solution containing metal ions. Differences were considered statistically significant at $P < 0.05$.

Analysis of aromatic compounds

By using the immobilized β-glucosidase, Glycosidic bound volatile compounds in mandarin fruit juice were isolated, and extracted with Amberlite XAD-2 resin, and then hydrolyzed by the immobilized β-glucosidase. The released glycosidic bound volatiles were analyzed by GC-MS and LC-MS. The samples extracted were analyzed with Shimadzu Gas Chromatography GC-2010 Plus. Compounds identified in the GC-MS (Gas Chromatography–Mass Spectrometry) analysis of the samples were determined by comparison with the Wiley229 and Nist27 mass spectroscopy libraries. The samples were analyzed in an electrospray ionization (Electrospray Ionization ESI) device with the Agilent LC-MS

(Liquid Chromatography–Mass Spectrometry) system.

Results and Discussion

Purification of β-glucosidase from mandarin

In this study, β-glucosidase was purified from mandarin fruits using an ammonium sulfate and Sepharose 4B-L-tyrosine-1-Napthylamine hydrophobic interaction column (Kara *et al.* 2011). Upon fractionation of the β-glucosidase active fractions with ammonium sulfate, 75% of the total activity was obtained in the fraction saturated with 40 – 80 % ammonium sulfate. To improve its binding efficiency, the precipitate with β-glucosidase activity was dissolved and saturated with 1.5 M ammonium sulfate before applying it to the sepharose-4B-l-tyrosine-1-napthylamine column. The fractions with the highest β-glucosidase activity and the relatively lower protein contents were pooled and used for enzyme assay. The fold purification and the enzyme yield (196.2 and 11 %) with the above procedure seem to be higher than with previous glucosidase purification studies from different plant sources (Gerardi *et al.* 2001; Odoux *et al.* 2003; Li *et al.* 2005; Yu *et al.* 2007). This is due to the favourable ammonium sulfate precipitation range and the specifically designed hydrophobic interaction column.

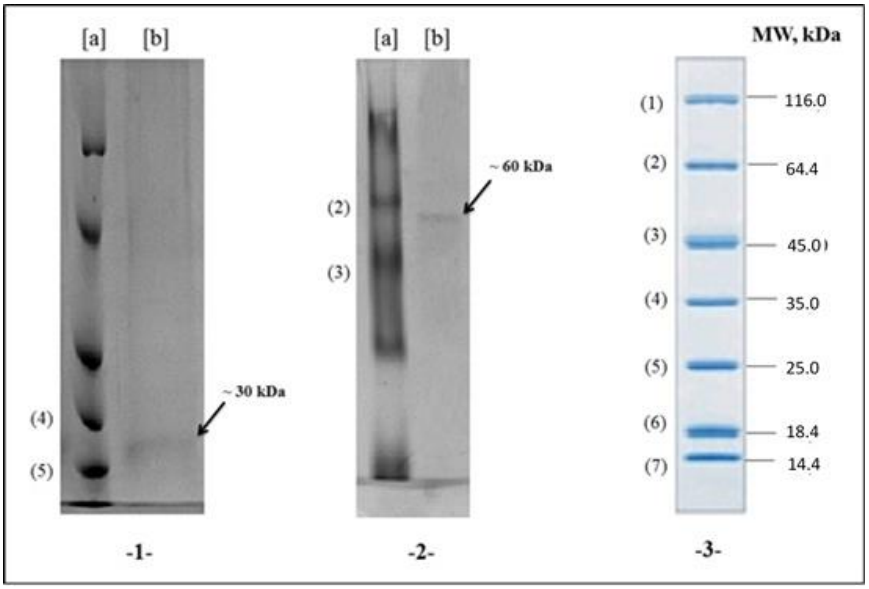

Fig. 1. SDS-Native PAGE of purified mandarin β-glucosidase. **1 –** SDS-PAGE; **2 –** Native-PAGE; **1a,2s,3** – Molecular Weight Marker (β-galactosidase, 116 kDa; bovine serum albumin, 66.4 kDa; egg albumin, 45 kDa; lactate dehydrogenase, 35 kDa; Rease Bsp981 (*E. coli*), 25 kDa; β-lactoglobulin, 18.4 kDa; lysozyme,14.4 kDa).

The purified β-glucosidase migrated as a single band during native and SDS–polyacrylamide gel electrophoresis when stained with Coomassie Brilliant Blue (Fig. 1). Mandarin β-glucosidase molecular weight was determined as 30 kDa and 60 kDa using SDS and native PAGE, respectively. Therefore, it was found that the enzyme had two subunits of equal size. The estimated molecular mass of the protein is a little smaller than β-glucosidases from various plant sources e.g., 64 kDa from orange (*Citrus sinensis*) fruit tissue (Cameron *et al.* 2001), 68 kDa from ripe sweet cherry (*Prunus avium*) fruit (Gerardi *et al.* 2001) and 62 kDa from *Sorghum bicolor* seedlings (Hosel *et al.* 1987).

Optimum binding capacity of the enzyme to nanoparticles (Binding efficiency)

The optimum binding percentage of the enzyme to the nanoparticles in the medium and activity were measured (Table 1). Using the data in Table 1, Fig. 2 was drawn, showing the relationship between the amount of nanoparticles and the percentage binding and activity of the β-glucosidase enzyme.

Table 1. Fe_3O_4 nanoparticle amount, mandarin immobilized β-glucosidase percentage, and activity.

Nanoparticle [mg]	Nanoparticle [mg.mL^{-1}]	Enzyme volume* [µL]	Protein amount [µg.mL^{-1}]	Protein amount in elution	Immobilized enzyme [%]	ΔA (405 nm)	Activity [U.mL.min^{-1}]
25	7.69	750 (*including 294,59 µg.mL^{-1} protein)	67.982	26.925	60.4	1.44	1850.813
50	15.38			-	100	1.47	1891.463
75	23.07			-		1.21	1539.160
100	30.76			-		1.15	1457.859
125	38.46			-		1.08	1363.008
150	46.15			-		1.02	1281.707

As shown in Table 1, it was found that 60.4 % of the enzyme was bound by 7.69 mg/mL Fe_3O_4 nanoparticle in the reaction medium containing 67.982 µg.mL^{-1} β-glucosidase enzyme. Besides the percentage of immobilized glucose, β-glucosidase increased and then remained at 100 % when the amount of nanoparticle added was above 15 15 mg.mL^{-1}. On the other hand, as the amount of Fe_3O_4 nanoparticles increased, the enzyme activity decreased (as is more clearly seen in Fig. 2). Therefore, the optimum Fe_3O_4 nanoparticle rate in the medium was revealed to be 15 mg.mL^{-1}.

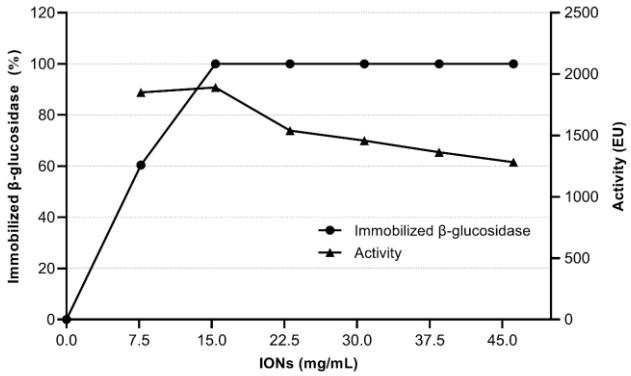

Fig. 2. Effect of the amount of IONs added on the percentage of immobilized β-glucosidase.

Particle size and structure

The XRD pattern of the IONs is given in Fig. 3. The nanoparticles have a cubic spinel structure with the characteristic (220), (311), (400), (422), (511), (440) and (622) peaks of iron oxide at around 2θ ≈ 31o, 35o, 43o, 53o, 57o, 63o and 74o respectively, according to the JCPDS cards no. 019-0629 and no. 039-1346. The mean crystal size of IONs, dXRD were calculated from the most intense peak (311) in the pattern using the Scherrer equation (Cullity 1978) and found to be 14.4 nm.

TEM images of the IONs were given in Fig. 4. The particles are mostly spherical. The physical particle size, dTEM, is found to be 9.0 ± 2.3 nm.

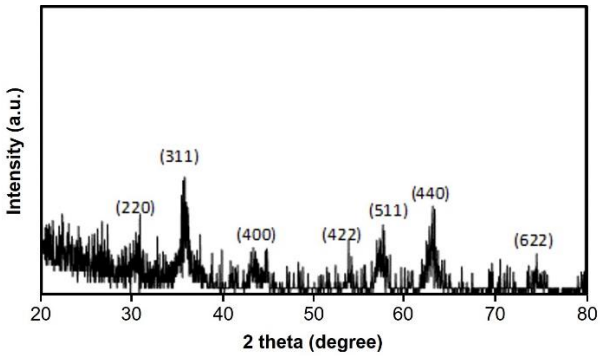

Fig. 3. XRD pattern of IONs.

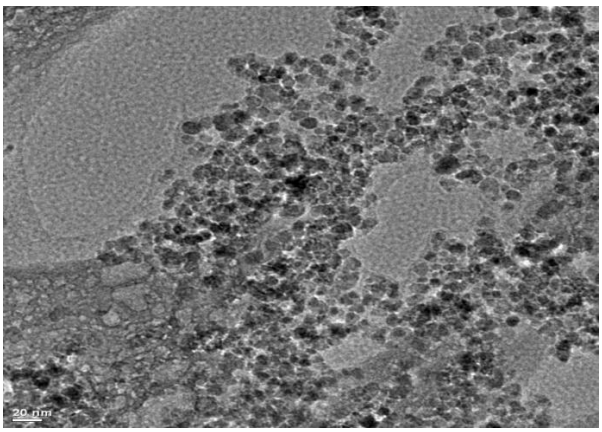

Fig. 4. TEM image of IONs.

Magnetic property

The magnetization curves of the IONs and β-glucosidase bound IONs were measured at ± 20 kOe and given in Fig. 5. The detailed curves drawn at ± 50 Oe are illustrated in the inset. IONs are superparamagnetic with zero coercivity, Hc. Saturation magnetization, Ms of the IONs is 73.6 emu/g. The mean magnetic particle size, dMAG (with standard deviation, σ) was calculated

according to the relations (Morales *et al.* 1999; Karaagac *et al.* 2010) and found to be 7.8 ± 2.4 nm. After the immobilization, the Ms Value of immobilized β-glucosidase is 56.3 emu.g^{-1} and the sample is superparamagnetic with a Hc of 3 Oe. About 24 % decrease in Ms was observed after the immobilization. This could be attributed to the binding of β-glucosidase to IONs, which leads to a reduction in the number of magnetic moments per weight in the volume fraction.

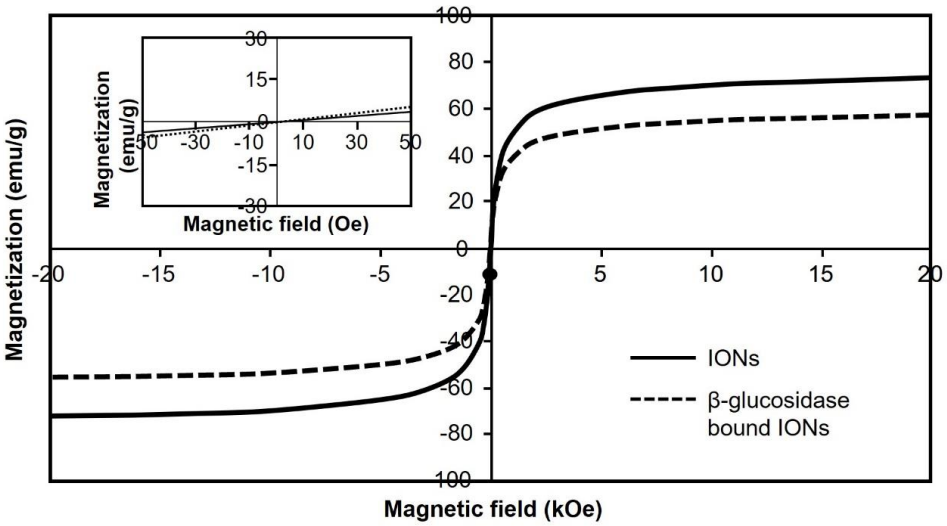

Fig. 5. Magnetization curves of IONs and β-glucosidase bound IONs (Inset shows the magnetization curves in ± 50 Oe).

Mechanism of binding

FTIR analysis was demonstrated for the β-glucosidase bound to magnetic nanoparticles (Fig. 6). FTIR spectra for the dried β-glucosidase from purified mandarin, IONs, and β-glucosidase bound IONs. The band around 538 cm^{-1} corresponds to the Fe-O vibration, confirming the sample is iron oxide. IONs nanoparticles were detected during a frequency peak at 538.2 cm^{-1}. The deepest peak frequency of 619.14 cm^{-1} observed in pure β-glucosidase shows a secondary amide group for the unique protein structure. It was evidence that the characteristic bands of protein (i.e., Liao and Chen 2001) at 1642 and 1631cm^{-1} were present in pure β-glucosidase and in the β-glucosidase-bound IONs, confirming the binding of yeast alcohol dehydrogenase (YADH) to IONs. The deep characteristic bands of proteins for the β-glucosidase-bound IONs should be due to the high enzyme loading.

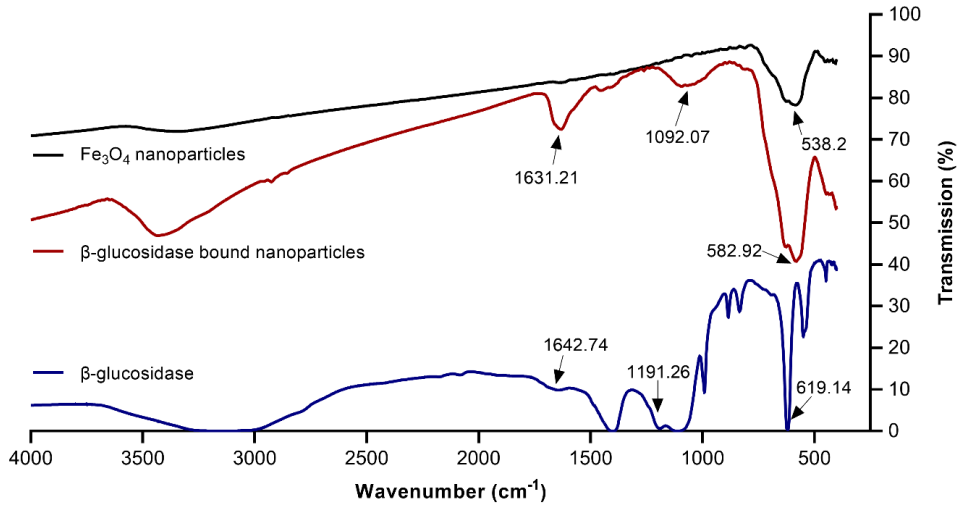

Fig. 6. FTIR analysis of nanoparticles, β-glucosidase and β-glucosidase bound nanoparticles.

Activity and stability

When the optimum Fe_3O_4 nanoparticle rate in the medium was 15 mg.mL^{-1}, because β-glucosidase was completely bound, the activities and stabilities of immobilized β-glucosidase were measured under this condition. As shown in Fig. 7A, optimum pH values for free and immobilized β-glucosidase were the same as in previous study (Su *et al.* 2010). However, the optimum temperature value of immobilized β-glucosidase was determined to be higher than free enzyme (50 ℃ and 40 ℃ respectively, Fig. 7B). As a positive result, the increase in the heat resistance of the mandarin β-glucosidase enzyme immobilized in the research is consistent with the results of Singh *et al.* (2011) It seems that immobilized β-glucosidase has generally more stability than the free enzyme.

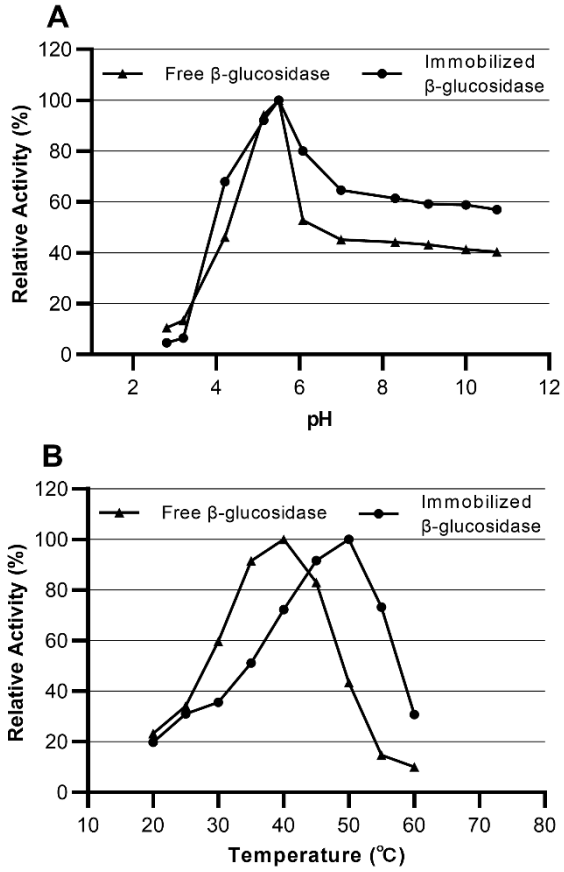

Fig. 7. Optimal pH (**A**) and temperature (**B**) for free and immobilized β-glucosidase.

Fig. 8 shows the storage stabilities of immobilized β-glucosidase and free β-glucosidase at 4 and 25 ℃ in a relative activity to time plot. After an incubation time of 30 days, the relative activities of free β-glucosidase at 4 and 25 ℃ were 74 and 23 %, respectively. However, the immobilized β-glucosidase retained 88 % and 47 % activity at 4 and 25 ℃ respectively, over a period of 30 days. It was observed that, the storage stability of the immobilized β-glucosidase significantly increased according to the free enzyme. Interestingly, it was determined that the immobilized β-glucosidase activities at both 4 ℃ and 25 ℃ were observed to be increased compared with the free β-glucosidase activity for 5 days. Similarly, many other studies (Fan *et al.* 2011; Chen *et al.* 2012; Su *et al.* 2010) reported that the immobilized enzyme was more stabled than the free enzyme.

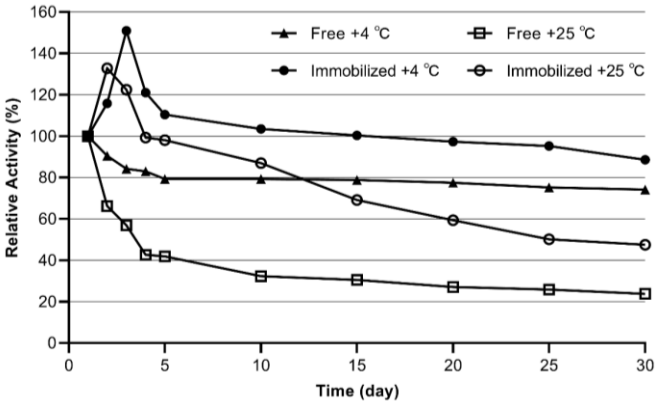

Fig. 8. Storage stability of the immobilized and free β-glucosidase at 4 ℃ and 25 ℃.

K_m and V_{max} values

The reaction kinetics of the immobilized and free β-glucosidase were calculated by using Lineweaver–Burk plots with the artificial substrate *p*-nitrophenyl-d-glucopyranosides (*p*-NPG) (Fig. 9).

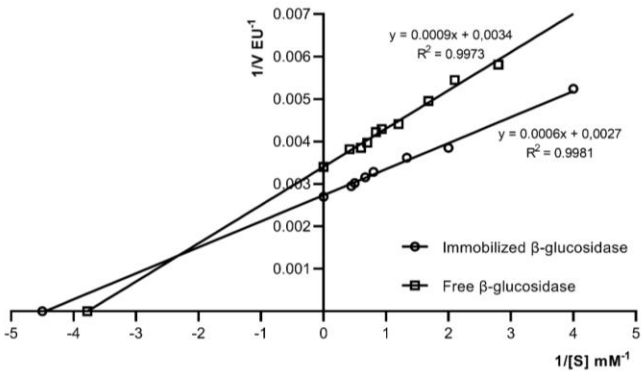

Fig. 9. Lineweaver–Burk plots of the immobilized and free β-glucosidase with *p*-NPG.

According to the plots, it was found that the K_m and V_{max} values of free and immobilized enzymes were 0.264 mM and 294 EU; 0.222 mM and 370 EU, respectively. Because of lower K_m values for immobilized enzyme, the affinity of the immobilized enzyme for *p*-NPG was higher than for free enzyme. As a result, after the immobilization, the rate of the enzyme catalytic activity increased and consequently, the K_m value of the β-glucosidase enzyme decreased. In other words, the immobilization process made the enzyme work more effectively. The higher immobilized activity has also been reported in others (Su *et al*. 2010; Fan *et al*. 2011).

The results of activity and stability are summarized in Table 2. As shown in the table, it can be revealed that immobilized enzyme would be very useful in industry.

Table 2. Activity and stability of β-glucosidase.

	Optimum pH	Optimum temperature [°C]	*p*-NPG V_{max} [EU]	*p*-NPG K_m [mM]	Storage stabilities 4 °C	25 °C
Free β-glucosidase	5.5	40	294.12	0.264	44 %	23 %
Immobilized β-glucosidase	5.5	50	370.97	0.222	88 %	47 %

The effect of various metal ions on the purified β-glucosidase enzyme was examined (Fig. 10).

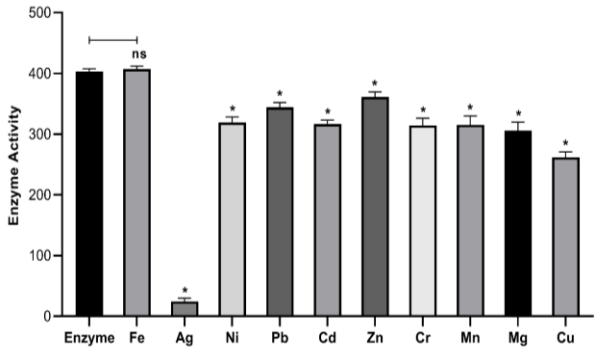

Fig. 10. Effect of some metal ions on β-glucosidase enzyme activity from mandarin (*$P \leq 0.05$, $^{ns}P > 0.05$).

Of the ions tested, only Fe^{2+} did not show an inhibitory effect on the enzyme activity, while others exhibited different levels of inhibitory effects. Similar results were reported in other studies (Cameron *et al.* 2001; Kara *et al.* 2011). According to the result, it can be said that it is very reasonable to use Fe^{2+} ions to immobilize the β-glucosidase enzyme.

Results of GC and LC analyses of aromatic compounds

The table below summarizes the analysis of mandarin juice hydrolyzed by immobilized β-glucosidase (Table 3).

Table 3. Compounds detected from mandarin juice hydrolyzed by immobilized β-glucosidase.

Compound	Analyze type	Retention time [RT, min]	Diagnostic Reference
Naringin	LC-MS (QN)	10.699	Calibrated Compounds
Hesperidin	LC-MS (QN)	11.116	Calibrated Compounds
Limonene	GC-MS (QL)	16.695	Wiley7 Lib.
Benzaldehyde	GC-MS (QL)	4.295	Wiley229 Lib.
Benzyl acetate	GC-MS (QL)	46.840	Wiley229 Lib.
1,3-Dimetil benzene	GC-MS (QL)	8.850	Wiley229 Lib.
Ethyl benzene	GC-MS (QL)	8.460	Nist27 Lib.
Benzophenone	GC-MS (QL)	44.580	Nist27 Lib.
2-Amino-1,3-propanediol	GC-MS (QL)	3.235	Nist27 Lib.
Phenol-2,6-bis (1,1 dimethylethyl)-4-methyl	GC-MS (QL)	39.485	Wiley7 Lib.

Abbreviations: QL Qualitative; QN, Quantitative

Naringin and Hesperidin were analyzed quantitatively, others qualitatively. When the chromatograms of the LC-MS analysis samples were examined, an increase was observed in the compounds that play a role in the aromatic quality of the fruit juice. The amount of hesperidin in the intact mandarin juice, which is at a level of 19.821 ppm, was determined at a level of 40.195 ppm in the sample treated with Fe_3O_4 nanoparticles and at a level of 74.23 ppm in the sample treated with immobilized β-glucosidase. While the amount of naringin was 116.74 ppm in intact fruit juice, it was found to be 365.771 ppm and 371.060 ppm in fruit juices treated with nanoparticles and immobilized β-glucosidase, respectively.

While no peak was observed in the GC-MS chromatograms of the fruit juice prepared as the control group and the juice treated with Fe_3O_4 nanoparticles, various peaks were observed in the juice chromatograms treated with immobilized β-glucosidase enzyme. These peaks were compared with the Wiley229 and Nist27 mass spectroscopy libraries, and limonene, benzaldehyde, benzyl acetate, 1,3-dimethyl benzene, ethyl benzene, benzophenone, 2-amino-1,3-propendiol, and phenol-2,6-bis (1,1-dimethylethyl)-4-methyl were detected. In other words, it was revealed that 8 compounds were formed by the immobilized β-glucosidase. The aroma enhancing effect of β-glucosidase enzyme immobilized by many researchers on orange juice (Fan *et al.* 2011), tea (Su *et al.* 2010), grape wine (Guengen *at al.* 1997; Pombo *et al.* 2011) and passion fruit juice (Shoseyov *et al.* 1990) was reported. It is the first time to be investigated the immobilization of β-glucosidase enzyme obtained from mandarin fruit on Fe_3O_4 superparamagnetic nanoparticles and its effect on the aromatic quality of fruit juice.

In conclusion, the present study has revealed the effectiveness of the purification procedure for β-glucosidase mandarin (*Citrus reticulata*). The enzyme was purified by salting it out with ammonium sulfate and using sepharose-4B-l-tyrosine-1-napthylamine hydrophobic interaction chromatography. The purified enzyme obtained from mandarin was immobilized onto the nanoparticles and characterized. Besides, it was determined that the immobilized enzyme had a flavor enhancing effect on mandarin juice.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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