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Enhancement of polyphenol bio-activities by enzyme reaction

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ABSTRACT

Using β-glucosidase to hydrolyze glycosides into aglycones, the present study attempted to improve the bio-activity of the extract from mulberry leaves. When varying the ethanol fraction, pH, and temperature of the extract, the optimum conditions for the enzyme reaction were identified as a 10% ethanol fraction in the extract, pH 5.0, and 40 °C temperature. Under these optimum conditions, the enzyme reaction produced a remarkable increase in the anti-oxidation and tyrosinase inhibition activities of the extract by as much as 219.5% and 230.9%, respectively. This improved bio-activity of the extract was due to the hydrolysis of the glycoside polyphenols rutin, isoquercitrin, and astragalin into the aglycone polyphenols quercetin and kaempferol. Furthermore, the enzymatic hydrolysis of the extract by β-glucosidase also produced some additional benefits that are critical factors for the skin absorption of bio-active ingredients, including an improved hydrophobicity (239.41%) and reduced mean molecular weight (from 387.3 to 291.4), resulting in a significantly enhanced skin permeability (513%).

1. Introduction

Recent studies on exploiting natural compounds for medicine and cosmetics have drawn much attention to the effective extraction of bio-active ingredients from natural products and maximization of the bio-activity of extracts for industrial application.

As a result, many attempts have already been made to enhance the extract efficiency of bio-active ingredients. Miguel et al. [1] investigated the optimum extracting solvent for anti-oxidant ingredients from among hexane, petroleum ether, water, and ethanol when varying the temperature. Plus, the optimum extraction conditions for polyphenols from wheat have also been suggested, along with the time, temperature, and mixture composition of methanol, ethanol, and acetone [2]. In a study by Kim et al. [3], it was shown that the optimal extraction conditions for solvents and their compositions depended on the polarity of the target bio-active ingredients. For example, in the case of mulberry leaves, anti-oxidation ingredients were optimally extracted when using high polar solvents, whereas a low polar solvent was preferred to extract tyrosinase inhibiting ingredients, implying that the optimal solvent conditions for the extraction of bio-active ingredients can be uniquely determined by matching the solvent polarity with the polarity of the target ingredient, regardless of the solvent species. Thus, the solubility parameter has been suggested as a single parameter for designing the optimal solvent for the extraction of specific bio-active ingredients.

In general, the low bio-activity of extracts has limited the practicability of their industrial application in cosmetics. Therefore, several attempts have been made to enhance the bio-activity of extract in various ways. The bio-active ingredients of plants existing in the form of glycosides are hydrophilic and soluble in water due to the glycosyl group. However, these properties of glycosides make them disadvantageous ingredients for skin cosmetics due to their low skin permeability. Meanwhile, aglycone ingredients are hydrophobic and can permeate human skin [4]. Thus, the hydrolysis of glycoside ingredients into an aglycone form has attracted attention as an effective way of enhancing the bio-activity of extracts [5–7]. Pandjaitan and Hettiarachchy [8] studied the enzyme reaction of β-glucosidase to hydrolyze genistin (genestein 7-*O*-β-D-glucopyranoside) into aglycone genestein based on varying the concentration, pH, and temperature. As a result, the genestein content was enhanced about 50%. In addition, quercetin-4′-glycoside and quercetin-3,4′-diglycoside were also used as ingredients to measure the effectiveness of the enzyme reaction, along with enzyme species, glycoside concentration, and reaction temperature [9]. Hydrochloric acid has also been used for the hydrolysis of genistin and daizin into genestein and daizein [10]. In this case, although the chemical hydrolysis is much faster than the enzymatic hydrolysis, an extra process is required to separate the hydrochloric acid before applying the product to human skin.

In previous studies, the focus has been limited to the enzymatic reaction of a single ingredient, where the hydrolysis of glycoside

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has been partially successful in enhancing the activity. However, the present study attempted to enhance the bio-activity of a natural extract using an enzyme. As distinct from previous studies, the enzyme reaction is directly applied to the extract that includes lots of ingredients, and the property enhancement then evaluated in terms of the bio-activities of anti-oxidation and tyrosinase inhibition for practical application. In addition, the influence of the enzyme reaction conditions on the bio-activity of the extract, such as the ethanol fraction in the extract, pH, and temperature, is also considered, along with the fundamental reasons for the enhanced bio-activity resulting from the enzyme reaction. Finally, for practical application in the cosmetic industry, the effect of the enzyme reaction on the hydrophobicity and skin permeability of the extract is examined.

2. Experiments

2.1. Mulberry leaf extraction

Mulberry (*Morus alba* L.) leaves purchased at a herbal market in Korea were completely dried in a convection oven at 60–80 °C for a couple of days and finely pulverized using a milling machine. The leaf powder was then sieved using a 200 μm aperture mesh and kept at 4 °C. To extract the bio-active ingredients, 2 g of the leaf powder was mixed with 20 ml of an ethanol (30%)–water (70%) solvent in a hot bath at 40 °C for 1 h. The leaf solid was then separated using a centrifuge (Hanil Science Industrial Co., HA-500, Korea) and the supernatant extract used for the enzyme reaction.

2.2. Enzyme reaction

Three milliliters of the mulberry extract was diluted with 7 ml of the ethanol–water solution and placed in an incubator for 6 h. The enzyme reactions were then initiated by the addition of 0.2 U/ml of β-glucosidase (Shin Nihon Chemical, Japan). To modify the enzyme reaction conditions, the ethanol fraction in the extract was changed from 5% to 80% (v/v) using pure water and ethanol, and the pH of the extract varied from 3.0 to 9.0 using acetate (from 3.0 to 6.0) and phosphate (from 6.0 to 9.0) buffers. Plus, the temperature of the enzyme reaction was also changed from 20 to 70 °C to determine the optimal conditions.

After 6 h, the extract was quickly mixed with 40 ml of methanol to quench the enzyme reaction and centrifuged at 3000 rpm for 20 min. The supernatant was then assayed for the bio-activities of anti-oxidation and tyrosinase inhibition.

2.3. Analysis of bio-activity

The anti-oxidation activity of the extract was evaluated based on the degree of scavenging 1,1-diphenyl-2-picryhydrazyl (DPPH) free radicals [11]. First, a free radical solution was prepared with 0.15 mM DPPH in 2 ml of ethanol and 0.1 ml of a 0.5% (v/v) Tween-20 solution. The pH of the radical solution was then adjusted to 7.4 with 1.8 ml of a 0.1 M Tris–HCl buffer. After adding 10 μl of the extract sample to the radical solution, the mixture was allowed to react for 30 min at room temperature, then the UV absorbance of the mixture was measured at a wavelength of 517 nm (JASCO, Model V-570, Japan). A blank solvent containing no extract was used as the base reference for the anti-oxidation activity. Plus, the anti-oxidation activity of the extract (C_{AO}) was defined using a relative scale of:

$$C_{AO}(\%) = \frac{A_{AO} - A_{RO}}{A_{RO}} \times 100 \quad (1)$$

where A_{AO} and A_{RO} are the UV absorbance of the extract and blank solvent, respectively.

Meanwhile, to analyze the tyrosinase inhibition activity, mushroom tyrosinase and L-dopa were purchased from Sigma Chemical Co. (ACS grade). Based on the method suggested by Lee et al. [12], the inhibition of the mushroom tyrosinase by the ingredients was measured as indicative activity of anti-hyperpigmentation. A 50 mM phosphate buffer solution (pH 6.8) was mixed with 0.96 ml of L-dopa (1 mM) and 0.32 ml of the extract, then 0.32 ml of the mushroom tyrosinase (125 U/ml) was added. The mixture was incubated at 37 °C for 10 min, then quickly cooled to 0 °C to quench the reaction. Thereafter, the UV absorbance of the mixture at 475 nm was measured. A blank solution containing no tyrosinase was used as the base reference for the tyrosinase inhibition activity. Plus, the tyrosinase inhibition activity of the extract (C_{AT}) was defined using a relative scale of:

$$C_{AT}(\%) = \frac{A_{AT} - A_{RT}}{A_{RT}} \times 100 \quad (2)$$

where A_{AT} and A_{RT} are the UV absorbance of the extract and blank solution, respectively.

2.4. Assay of polyphenols

Based on the method of Goldstein and Swain [13], the amount of polyphenols in the extract was evaluated. First, the extract was diluted 50 times with distilled water and 0.1 ml of the diluted sample completely mixed with 1.25 ml of the Folin-Denis reagent (ACS grade, Fluka, Switzerland) that had been diluted 10 times with distilled water. Thereafter, 0.25 ml of saturated sodium carbonate was added and the mixture incubated at 25 °C for 20 min. Using a UV spectrophotometer at a 760 nm wavelength, the amount of polyphenols was then estimated based on comparison with a standard concentration curve prepared using tannic acid. The standard concentration of tanic acid was also prepared based on the same procedure as described above for the extract. Thus, 0.1 ml of a tannic acid solution was mixed with 1.25 ml of the Folin-Denis reagent and 0.25 ml of saturated sodium carbonate, followed by incubation at 25 °C for 20 min. The UV absorbance of the tannic acid solution relative to the concentration produced a standard curve as follows:

$$C_P(\%) = \frac{A_{AT} - 0.10467}{0.09488} \times 100 \quad (3)$$

where C_p is the tanic acid concentration (g/l) and A_{AT} is the UV absorbance of the tanic acid solution. Eq. (3) was then used to estimate the amount of polyphenols relative to the amount of tannic acid.

2.5. Measurement of hydrophobicity

The hydrophobicity of the extract was estimated in terms of the *n*-octanol/water partition coefficient (K_{ow}) according to the shake flask method [14]. A polyphenol sample dried from 10 ml of the extract was dissolved with 40 ml water and added to 20 ml of *n*-octanol in a bottle. The mixture was then rigorously shaken for 1 h to reach an equilibrium phase. Thereafter, the *n*-octanol phase was separated from the water phase using a centrifuge (Hanil Science Industrial Co., Ltd. HA-500, Korea), and the polyphenol concentrations measured in each phase. The octanol/water partition coefficient was then calculated as follows:

$$K_{OW} = \frac{C_O}{C_W} \quad (4)$$

where C_O and C_W are the polyphenol concentrations in the *n*-octanol and water phase, respectively.

2.6. HPLC analysis

The quercetin and rutin in the extract were analyzed using an HPLC (Agilent, 1100 series, U.S.A.) equipped with a quaternary pump, UV detector at 363 nm, and Eclipse XDR-C_{18} reversed-phase column (15 cm × 4.6 mm i.d., 5 μm particle size). The mobile phase used a methanol–acetonitrile–water (40:15:45, v/v/v) mixture containing 1.0% acetic acid, where the flow rate and sample injection volume were fixed at 0.5 ml/min and 20 μl, respectively. As reference ingredients, pure quercetin and rutin were purchased from Sigma–Aldrich (ACS grade, U.S.A.).

3. Results and discussion

3.1. Effect of enzyme reaction conditions

An organic solvent is important for the effective extraction of the bio-active ingredients from mulberry leaves. Based on a previous study by Kim et al. [3], the optimal solvent conditions for extraction were identified as an ethanol (30%)–water (70%) mixture. However, since the alcohol species in the raw extract has an influence on the enzyme reaction, the alcohol fraction in the extract was adjusted using pure ethanol and water to produce an optimal enzyme reaction. Thus, to obtain an ethanol fraction below 30%, the raw extract was diluted with water, whereas pure ethanol was added to obtain an ethanol fraction higher than 30%. An enzyme reaction with β-glucosidase was then applied to the adjusted extract to enhance the bio-activity, which was quantitatively evaluated based on the ratio of the activity in the extract before and after the enzyme reaction. A ratio greater than 100% indicated enhanced bio-activity in the extract following the enzyme reaction.

As shown in Fig. 1, the anti-oxidation activity of the extract was enhanced by the enzyme reaction across the entire composition range of ethanol. In particular, with a low ethanol fraction below 30%, the anti-oxidation activity was enhanced up to 200% when decreasing the ethanol fraction. Conversely, the anti-oxidation enhancement was significantly retarded when increasing the ethanol fraction in the extract above 40%, with the β-glucosidase enzyme seemingly completely deactivated with an ethanol fraction of 80%. This deactivation of β-glucosidase according to the ethanol fraction in the extract also had a significant effect on the tyrosinase inhibition activity. Thus, the enzyme reaction in a diluted extract with a 5–10% ethanol fraction resulted in a 250% enhancement of the tyrosinase inhibition activity. However, this enhancement was rapidly reduced when increasing the ethanol fraction, and almost disappeared with an ethanol fraction above 40%.

It should be noted that the enzyme reaction was also conducted with the raw extract to compare to the results with those for the adjusted extract. Therefore, the raw extract obtained with a 10% ethanol fraction solvent was directly treated with the β-glucosidase enzyme, and the resulting bio-activity of the raw extract compared with that of an extract adjusted to a 10% ethanol fraction from a raw extract obtained from a 30% ethanol fraction solvent. The comparison demonstrated that conducting the enzyme reaction with the raw extract was essentially ineffective as regards enhancing the bio-activities, as the 10% ethanol fraction solvent was disadvantageous for the extraction of the bio-active ingredients from the mulberry leaves.

In Fig. 2, the variation in the bio-active ingredients of rutin and quercetin in the extract is tracked relative to the ethanol fraction. Rutin (quercetin 3-*O*-rutinoside), as a glycoside polyphenol, is one of the most characteristic ingredients involved in the anti-oxidation activity of mulberry leaves. In the case of hydrolysis by β-glucosidase, the rutin (2.42 on scale of TEAC (Trolox Equivalent Anti-oxidant Capacity)) in the extract was converted into aglycone quercetin (4.72 on TEAC scale) [4,15,16], and this enzymatic conversion was maximized with a 10% ethanol fraction in the extract. However, no enzymatic conversion of rutin into quercetin was achieved when the extract included an ethanol fraction above 40%. These rutin and quercetin profiles relative to the ethanol fraction in the extract (Fig. 2) were also consistent with the anti-oxidation and tyrosinase inhibition profiles (Fig. 1).

It should be mentioned that the glycosides rutin and astragalin (kaemferol 3-*O*-glucoside) are active for anti-oxidation, yet not for tyrosinase inhibition, whereas the aglycones quercetin and kaemferol are highly active for both anti-oxidation and tyrosinase inhibition. Therefore, the enhanced bio-activities resulting from the enzyme reaction were primarily due to the hydrolysis of the glycoside polyphenols into aglycone polyphenols.

The effect of pH on the enzyme reaction in the extract is shown in Fig. 3. According to the profile of tyrosinase inhibition activity, the optimum pH condition for the enzyme reaction was around 5.0, even though pH 3.5 is known as the optimal pH for

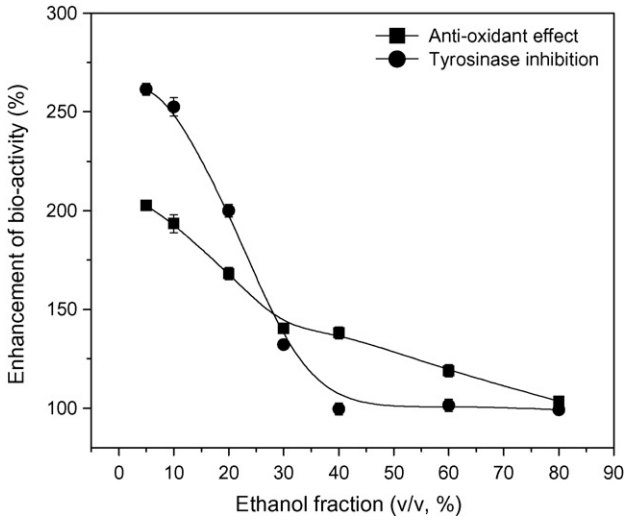

Fig. 1. Variation of bio-activity of anti-oxidation and tyrosinase inhibition in extract with ethanol fraction. Other enzyme reaction conditions were fixed at 40 °C and pH 5.0.

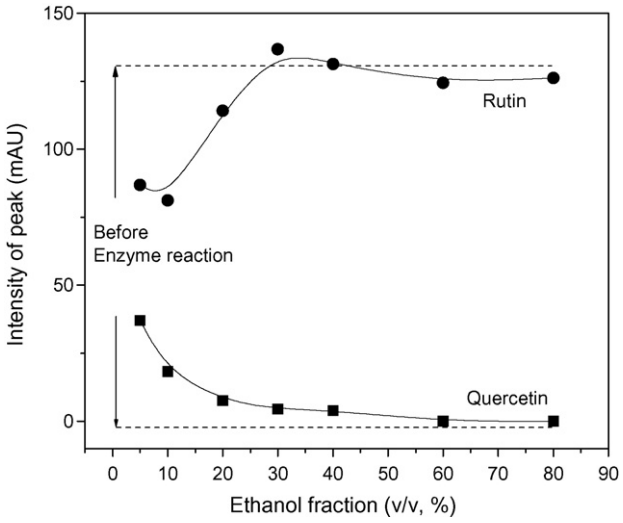

Fig. 2. Variation of rutin (●) and quercetin (■) concentrations in extract with ethanol fraction. Other enzyme reaction conditions were fixed at 40 °C and pH 5.0.

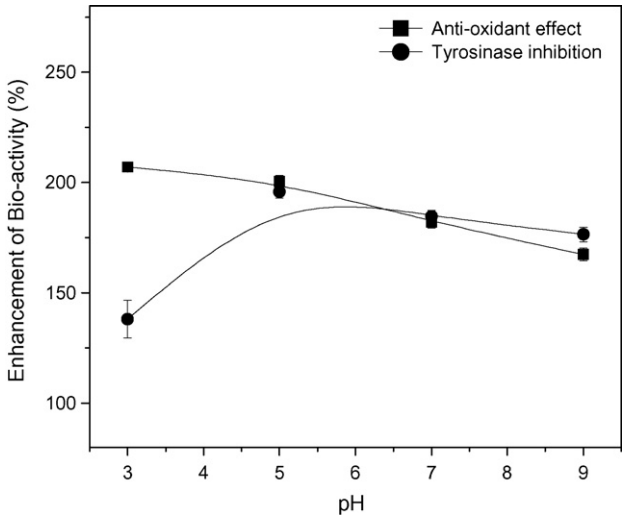

Fig. 3. Variation of bio-activity of anti-oxidation and tyrosinase inhibition in extract with pH. Other enzyme reaction conditions were fixed at 40 °C and 10% ethanol fraction of extract.

the enzyme activity of β-glucosidase [17]. Meanwhile, the anti-oxidation activity in the extract monotonically decreased when increasing the pH. This shift in the optimal pH condition for the enzyme reaction may have been due to proteins in the extract, which were simultaneously extracted by the solvent along with the polyphenols from the mulberry leaves, thereby contributing to the bio-activities. The presence of proteins in the raw extract was in fact confirmed using SDS-PAGE, as shown in Fig. 4(a). Therefore, the proteins in the raw extract were simply removed by ultrafiltration (10 kDa-polyethersulfone membrane, Millipore, U.S.A.), allowing the contribution of the proteins to the bio-activity of the raw extract to be estimated based on a comparison of the bio-activities in the raw extract and the ultrafiltered extract, as shown in Fig. 5. As a result, about 55% of the tyrosinase inhibition in the raw extract was found to originate from the proteins, whereas they only contributed to about 25% of the anti-oxidation activity. Consequently, since the proteins had an apparently significant impact on the tyrosinase inhibition of the extract, it is reasonable to suppose that the pH dependency might differ from that for β-glucosidase, thereby explaining the deviation in the optimum pH (pH 5.0) for tyrosinase inhibition from the optimal pH (pH 3.5) for β-glucosidase activity.

The influence of temperature on the enzyme reaction was also investigated, as shown in Fig. 6. When increasing the temperature, the anti-oxidation and tyrosinase inhibition in the extract were improved by the enzyme reaction due to an increase of enzyme activity. However, after passing 40 °C, both bio-activities were reduced, even though the enzyme activity of β-glucosidase is known to be maximized at a high temperature of 58 °C.

According to Qi et al., Matsufuji et al., and Inami et al. [18–20], the bio-active ingredients of polyphenols and antocynin extracted from hawthorn and radish quickly degrade at a high temperature above 40 °C. Similarly, in the present study, it would seem that the thermal degradation of the bio-active polyphenols at a high temperature above 40 °C surpassed the enzymatic conversion of the glycoside polyphenols into aglycone polyphenols in the extract, thereby diminishing the enhancement of the anti-oxidation and tyrosinase inhibition by the enzyme reaction. In addition, the proteins in the extract may also have been deactivated at the high temperature, resulting in the greater reduction of the tyrosinase inhibition compared to the anti-oxidation when increasing the temperature above 40 °C.

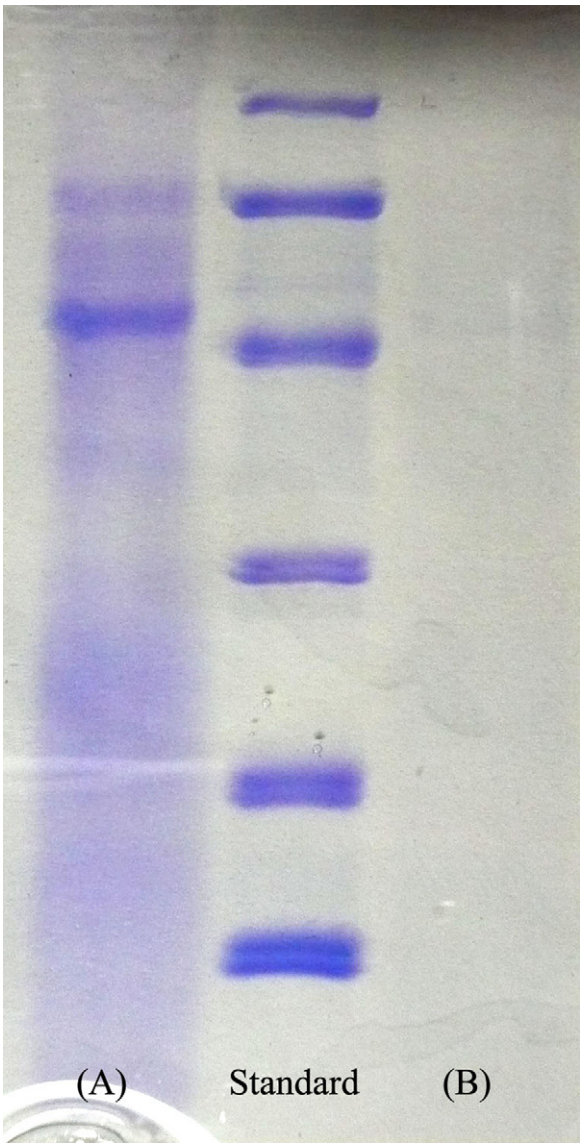

Fig. 4. SDS-PAGE of (A) raw extract and (B) raw extract filtered by UF membrane (10 kDa).

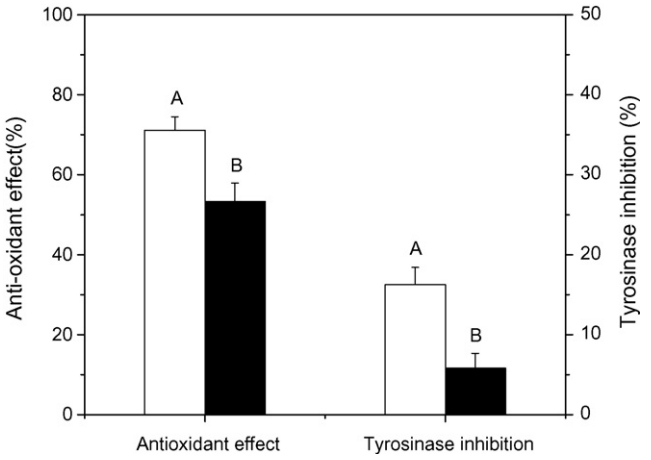

Fig. 5. Variation of bio-activity of anti-oxidation and tyrosinase inhibition of (A) raw extract and (B) raw extract filtered by UF membrane (10 kDa).

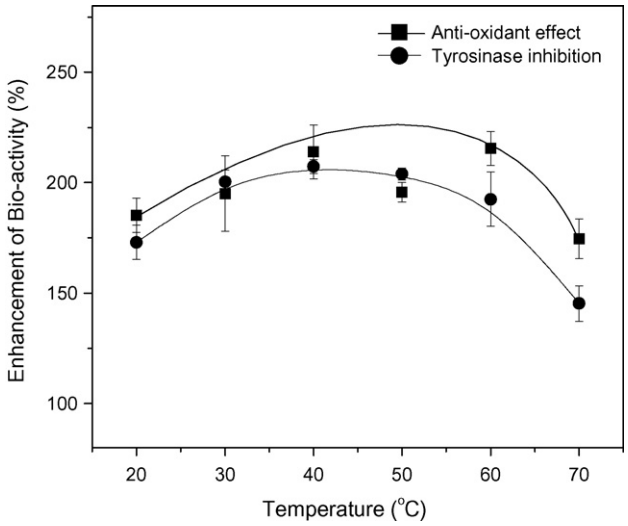

Fig. 6. Variation of bio-activity of anti-oxidation and tyrosinase inhibition in extract with temperature. Other enzyme reaction conditions were fixed at pH 5.0 and 10% ethanol fraction of extract.

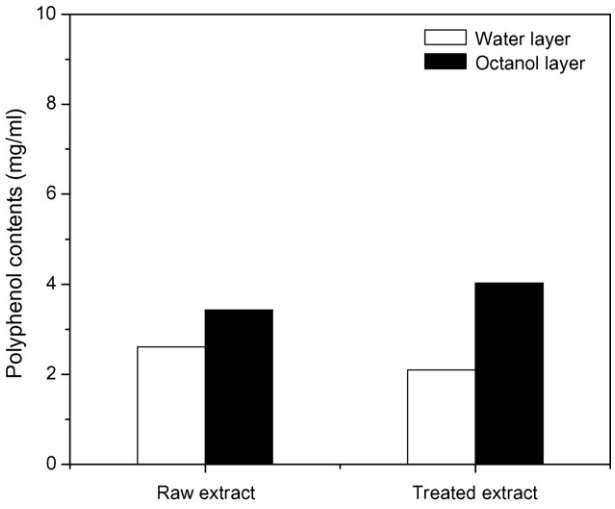

Fig. 8. Concentration of polyphenols in octanol and water phases.

3.2. Characteristics of extract

Based on the optimal conditions identified for the enzyme reaction (10% ethanol fraction in the extract, pH 5.0, and 40 °C), the bio-activities of anti-oxidation and tyrosinase inhibition in the extract were improved by as much as 219.5% and 230.9%, respectively. Therefore, the changes in the characteristic properties of the extract, such as the hydrophobicity and skin permeability, were also investigated as important factors for cosmetic application.

Using the contact angle method, the variation in the hydrophobicity of the extract was measured according to the ethanol fraction in the extract. In general, aglycones have a strong hydrophobicity, whereas glycoside polyphenols are highly hydrophilic due to the glucosyl group. Thus, an increase in the contact angle of the extract as a result of β-glucosidase may indicate a higher conversion of glycoside polyphenols into aglycone polyphenols, thereby further enhancing the bio-activity. As shown in Fig. 7, the hydrophobicity of the extract was significantly improved by the enzyme reaction with a low ethanol fraction of 5% and 10% in the extract, however, the hydrophobicity rapidly diminished when increasing the ethanol fraction. This also matched the variation of the bio-activity in the extract relative to the ethanol fraction (Fig. 1). Here, the enhancement of the hydrophobicity was defined as the percent ratio of the contact angles for the extract before and after the enzyme reaction.

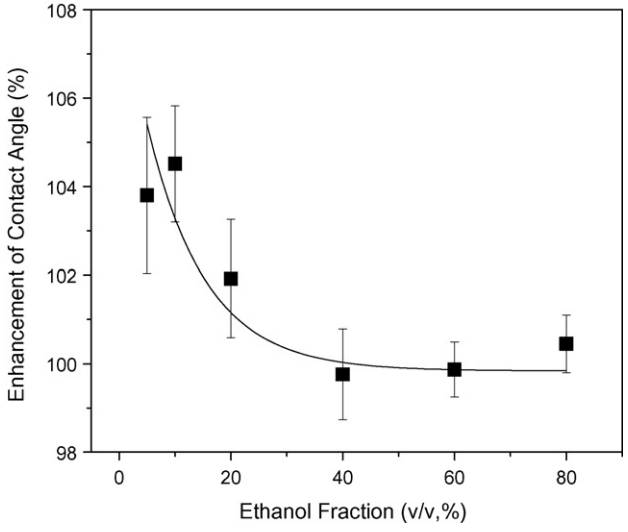

Fig. 7. Variation of contact angle in extract with ethanol fraction. Other enzyme reaction conditions were fixed at 40 °C and pH 5.0.

Using Potts and Guy's method [21,22], the skin permeability (K_p) of the bio-active ingredients in the extract was evaluated as follows:

$$\log K_p\,(\mathrm{cm\,s^{-1}}) = -6.3 + 0.71 \log K_{ow} - 0.0061\,\mathrm{MW} \quad (5)$$

where the octanol–water partition coefficient (K_{ow}) of the extract was estimated using Eq. (4) and the average molecular weight of the ingredients in the extract was measured with an LC–mass spectroscopy (JEOL, JMS-AX 505WA, Japan).

As shown in Fig. 8, in the case of the extract before the enzyme reaction, the polyphenol content in the octanol phase (C_O = 3.4 mg/ml) was slightly higher than in the water phase (C_W = 2.6 mg/ml), resulting in a 1.31 partition coefficient. However, after the enzyme reaction, the partition coefficient was enhanced to 2.05, as the polyphenol content in the octanol phase increased to 4.1 mg/ml, whereas that in the water phase was reduced to 2.0 mg/ml. These results confirmed the enzymatic hydrolysis of glycosides into aglycones in the extract, resulting in an increased hydrophobicity.

An analysis of the mean molecular weight of the ingredients in the extract is presented in Fig. 9. Before the enzyme reaction, the reference molecular weight for the ingredient with the highest peak intensity was 365 (m/z), and the relative peak intensities of the ingredients were distributed in broad range of the high molecular weight. However, after the enzyme reaction, the reference molecular weight for the ingredient with the highest peak intensity shifted to 203 (m/z), while the molecular weight distribution of the ingredients was obviously shifted to the left, as the high molecular weight glycoside polyphenols were hydrolyzed into low molecular weight aglycones and glucose by β-glucosidase. As a result, the mean molecular weight (MW) of the ingredients in the extract was reduced from 387.3 to 291.4.

Thus, when applying the partition coefficients and mean molecular weights to Eq. (5), the skin permeability coefficient ($\log K_p$) for the extract was confirmed to have been enhanced from −8.58 to −7.87 by the enzyme reaction, corresponding to a 513% enhancement of the skin permeability.

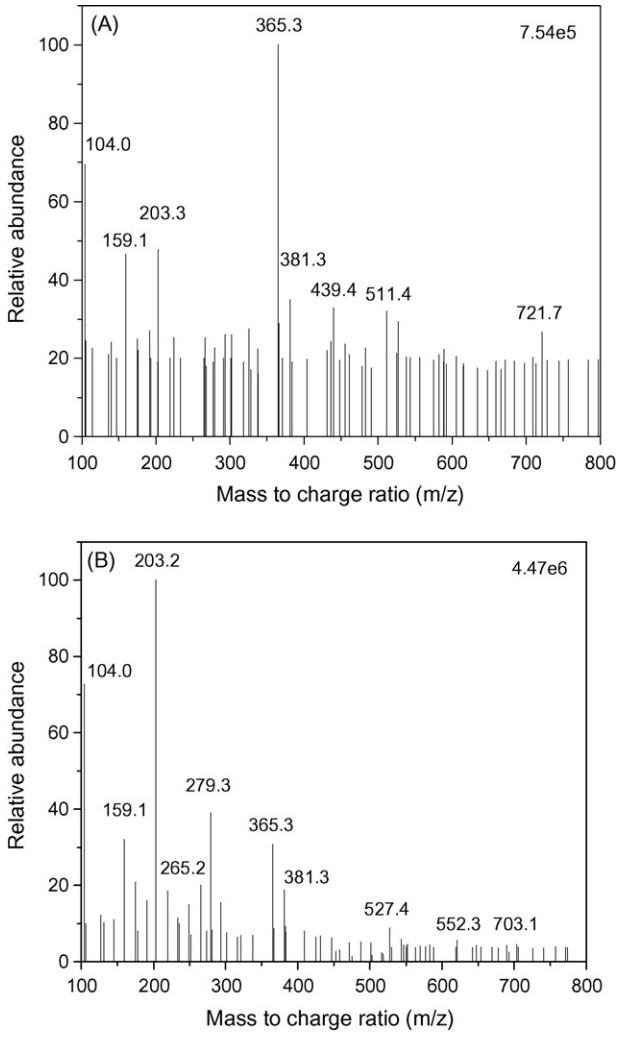

Fig. 9. Molecular weight distribution of ingredients (a) in extract before enzyme reaction and (b) in extract after enzyme reaction.

4. Conclusion

The enzymatic treatment of a natural extract was proven as an effective way to enhance the bio-activities of anti-oxidation and tyrosinase inhibition. Using β-glucosidase, the anti-oxidation and tyrosinase inhibition activities were improved up to 219.5% and 230.9%, respectively, as the glycoside ingredients were converted into highly active aglycones. As a result of varying the enzyme reaction conditions, a solvent with a 30% ethanol fraction was found to be optimal to extract the bio-active ingredients from mulberry leaves, whereas diluting the raw extract to include a 10% ethanol fraction was identified as most effective for the enzyme reaction. In addition, although the optimal pH for the enzyme activity of β-glucosidase is 3.0, the optimal reaction condition for the bio-activity of the extract was about pH 5.0, as proteins extracted from the mulberry leaves also contributed to the tyrosinase inhibition. The optimal temperature for the enzyme reaction was 40 °C, due to serious degradation of the bio-active polyphenols above a temperature of 40 °C.

As a result of the enzyme reaction, the hydrophobicity and skin permeability of the extract were both improved. The enzymatic hydrolysis of the hydrophilic glycosides into hydrophobic aglycones increased the hydrophobicity of the extract and decreased the mean molecular weight of the ingredients in the extract from 387.35 to 291.42. Consequently, the skin permeability of the bio-active ingredients in the extract was enhanced about 513%.

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