Enhancement of Natural Pigment Extraction Using *Bacillus* Species Xylanase

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Pigment extracts from the root of *Lithospermum erythrorhizon* are used as natural red dyes, as well as basic drugs due to their numerous pharmacological activities. In recent years, the demand for such natural pigment materials has increased; however, in natural dye production, the pigment yield is strongly affected by the source of cultivation, extracting conditions, and solvents. Accordingly, this study proposes a method of enzymatic pigment production based on the introduction of hydrolytic enzymes prior to the usual extraction to avoid repeated pigment extraction. The matrix destruction in the epidermal layer of the root by the enzymes was found to improve the pigment extractability, that is, the increment of K_L, the mass transfer coefficient, representing the pigment mobility in the epidermal layer. The root tissue maceration by the hydrolytic enzymes was also measured to evaluate the pigment extractability, and a linear relationship was observed between the K_L values and the tissue maceration up to the addition of 3000 units/g of xylanase, indicating that the enzymatic maceration proportionally increases the interfacial area between the pigment and the solvent. *Bacillus* sp. DX107 xylanase only served to increase the extractability of the pigment by loosening the root shell matrix, without affecting the contents and color properties of the pigment, as almost no difference was found in the color between the pigments extracted using xylanase and those extracted according to the traditional method.

KEYWORDS: *Lithospermum erythrorhizon*; xylanase; mass transfer coefficient; pigment extraction

INTRODUCTION

Extracts from the roots of *Lithospermum erythrorhizon* in eastern Asia have long been used as natural red dyes and crude drugs (*1*). The important components in the extracts are alkannin and shikonin, which are currently used for food coloring and cosmetics (*2, 3*). The biological activities of the extracts have also been widely reported (*4*). In particular, it is supposed that the products from the outer surface of the roots in the Boraginaceae family possess a broad spectrum of significant biological activities, including anti-inflammatory, antibacterial, antifungal, immunostimulating, anticancer, analgesic, and anti-pyretic activities (*5, 6*). Thus, because numerous biological activities have been revealed, use of these natural coloring products (or the extracted materials) is increasing gradually.

To produce a natural red dye, many root samples have been taken from different locations and studied using various kinds of solvent. However, because the concentration of dye com-

pounds in the outer root surface is strongly affected by the extraction conditions and solvent, and the pigment quantity varies from study to study, a standardized and efficient method for pigment extraction is needed (*7*). As such, an efficient extraction process involving nonpolar products—dyes with a low aqueous solubility—should increase and maintain the mass transfer rate value (*8*).

The mass transfer rate is calculated on the basis of three major factors: the product of the mass transfer coefficient (K_L), interfacial area per unit volume (*a*), and the concentration driving force. The concentration driving force is typically constant owing to the inherent solubility of the solutes. Therefore, repeated extraction is necessary for the efficient production of pigments from the plant root shell, where a repeated solvent change maintains the concentration driving force between the solute and the solvent. In addition, the *a* value can be increased by grinding the outer surface of the roots. Mechanical agitation is commonly used in mass-transfer-limited systems. However, these approaches are energy-intensive and thus, costly, particularly for large-scale systems (*9*). Although K_L varies somewhat with the environmental conditions and its range is relatively small, another approach is to increase the mass transfer

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coefficient using polysaccharide-degrading enzymes to macerate the extracellular matrix of the tissue prior to extraction in the organic phase (*10, 11*). In wine production, maceration refers to the breakdown of grape solids following grape crushing and is always included in the initial phase of red wine production. However, there have been insufficient studies on the relationship between the quantity of the wine quality-enhancing compounds and the effect of the added enzymes (*12*). The current authors previously reported that the hydrolytic enzymes produced by microorganisms were helpful to screen biologically active phytochemicals and that their biological properties could be enhanced by removing saccharides (*13*).

Accordingly, this study evaluates the efficiency of cell wall-degrading enzymes for natural dye production from the epidermal layer of the root of *L. erythrorhizon*. As such, the mass transfer coefficient is measured to evaluate the extractability of the pigments, plus the difference in the color characteristics of the pigments extracted using the enzymes is examined.

MATERIALS AND METHODS

Enzyme Preparation and Estimation of Enzymatic Hydrolysis. *Bacillus* sp. DX107 isolated from Korean soil was used for the production of xylanase. The DX107 was grown for 48 h at 60 °C in 3.25 L of an AG medium, containing 0.5% arabinogalactan from sugar beet pulp as the sole carbon source, 0.2% ammonium sulfate, 0.6% KH_2PO_4, 1.4% K_2HPO_4, and 0.02% yeast extract (Difco, Detroit, MI) (separately autoclaved at pH 7.0), using a KF-5L fermentor (Korea Fermentor Co., Seoul, Korea). The cultures were centrifuged at 2780*g* for 15 min at 4 °C to permit sedimentation. The supernatant was then concentrated to 500 mL in a Pellicon ultrafiltration system (Millipore Corp., Bedford, MA) using a 10000-Da molecular mass cutoff polysulfone filter. The extracellular xylanase was precipitated at 0 °C with ammonium sulfate (80% saturation) and separated by sedimentation at 4 °C overnight. After centrifugation at 4360*g* for 15 min, the dissolved pellet was dialyzed and used as crude xylanase for the experiments. The other enzymes were purchased from Sigma Chemical Co. (St. Louis, MO).

The hydrolytic activity of each enzyme was measured from the amount of soluble reducing sugars in the supernatant after centrifugation of the digestion mixture after incubation with each appropriate substrate. A ferricyanide technique was adopted from the methods of Kidby and Davidson (*14*), and a standard curve was obtained using a glucose solution with a known concentration.

Root Tissue Maceration. The root tissue maceration was measured using stem tissue from an *L. erythrorhizon* root according to the method of Mussell and Morre (*15*). Cylinders were cut and removed from the stem root tissue using a cork borer (0.5-mm diameter). The root shells were then sliced into 5-mm lengths and immediately immersed in distilled water. After standing for 1 h, the shells were gently blotted onto filter paper to remove the excess water, dried at 80 °C for 2 h, and weighed to the nearest milligram. To measure the degree of tissue maceration by the enzymes, the root shell was incubated with each enzyme at a concentration of 1000 units/g. After incubation for 1 h at 37 °C while shaking at 80 rpm, the root shells were vortexed at maximum speed for 20 s, then poured onto a concave wire mesh filter (≥0.8-mm mesh size) to remove the loosened cells. The resultant root shells were then blotted gently with filter paper, dried at 80 °C for 2 h, and weighed to the nearest milligram. The maceration was expressed as the weight loss of each sample after incubation with the enzymes and calculated per 100 mg of the starting tissue. Three replications were used for the various hydrolytic enzymes, and the data reported were normalized to the activity of untreated samples.

Pigment Extraction and Pigment Yield Measurement. Sliced dry root shells of *L. erythrorhizon* were hydrolyzed with various hydrolytic enzymes (3000 units/g) in a 50 mM appropriate buffer solution. The hydrolysates were filtered through Whatman no. 2 paper and dehydrated at room temperature. The dried filtrates were then extracted with methanol at room temperature in an inert (nitrogen) atmosphere, and the methanol-extracted fraction was concentrated in vacuo and weighed.
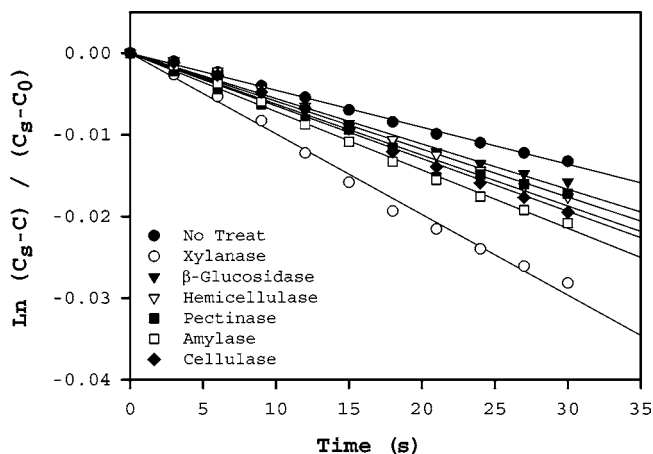

Figure 1. Agreement between experimental data and linear trend predicted by eq 3.

A liquid chromatographic system with a diode array detector was used to analyze the pigment and measure the pigment production. Each sample was centrifuged at 9860*g* for 15 min, and the supernatant was blown with N_2 gas until dry. The residue was then reconstituted with 1 mL of methanol and subjected to an HPLC analysis. The chromatographic system was composed of a Hitachi ELITE LaChrom HPLC system equipped with L-2130 pumps and an L-2450 diode array detector at an operative wavelength from 200 to 600 nm. For the spectral acquisition and evaluation, an EZ Chrom Elite v 3.1.3 (Hitachi high-technology) was used. The separations were performed on a 5-μm YMC-Pack Pro C_{18} (4.6 × 250 mm, YMC, Tokyo, Japan) column. The mobile phases used to analyze the various samples were mixtures of 0.1% (v/v) phosphoric acid (A) and acetonitrile (B) with an isocratic or gradient elution, where the gradient elution was 20−100% B within 30 min at a flow rate of 1.5 mL/min.

Mass Transfer Measurement. The un-steady-state mass balance of the pigment contents in the continuous phase was calculated using the following equations:

$$V_c \frac{dC}{dt} = K_L aV(C^* - C) \qquad (1)$$

Equation 1 was then integrated from the initial time (t_0) and concentration (C_0) to obtain

$$\ln\left[\frac{(C^* - C)}{(C^* - C_0)}\right] = -\left[K_L a \frac{V}{V_c}\right](t - t_0) \qquad (2)$$

The experimental data were analyzed by plotting the left-hand side of eq 2 as a function of time. The value of $K_L a$ was then obtained from the slope of the plot using the relationship

$$K_L a = -\text{slope}\left[\frac{V_c}{V}\right] \qquad (3)$$

For each of the seven types of mass transfer experiments performed, plots showing the degree to which the data followed the expected linear trend are given in **Figure 1**. The other parameters were obtained experimentally: *a*, 644.76 mm^2; *C**, 24.46 mg; *V*, 60.77 mm^3.

Hunter Values and Reflectance Measurements. The Hunter values and reflectance spectra of the pigment solutions were assessed using a colorimeter (JS555, Japan) at a 5-nm resolution over a wavelength range from 400 to 700 nm to approximate the range of human color vision. The absorbance measurements were made using a standard double-beam arrangement, with the absorption of the pigment solution measured relative to that of a reference cell containing distilled water. Meanwhile, the spectral reflectance of the emulsions was measured relative to a barium sulfate ($BaSO_4$) standard white plate.

RESULTS AND DISCUSSION

Pigment Extraction from Root of *L. erythrorhizon*. Because the extract from the roots of *L. erythrorhizon* was not originally

Development of Enzymatic Pigment Extraction

J. Agric. Food Chem., Vol. 53, No. 7, 2005 **2543**

Table 1. Pigment Yield and Hunter Values[a] for *L. erythrorhizon* Pigments Using Various Solvents

solvent	weight[b] (g)	L	a	b
water	0.01	73.47	6.87	14.89
methanol	1.28	57.87	41.37	15.78
acetonitrile	0.85	58.88	41.79	13.49
ethyl acetate	0.65	59.02	42.24	13.40
acetone	1.05	58.15	43.87	12.23
hexane	0.35	62.79	43.78	5.68
dichloromethane	0.78	64.39	44.75	4.34

[a] Hunter values indicate a point plotted in the color space organized in a cube form; *L* value, whiteness; positive/negative *a* value, red/green; positive/negative *b* value, yellow/blue. [b] Pigment weight was measured from extracts of 100 g of *L. erythrorhizon.*

Figure 2. Pigment yield from extraction after pretreatment with various hydrolytic enzymes.

known as a dyeing material but rather for its wound-healing properties, it was naturally obtained for a long time and mainly extracted using hot water. The biological properties of the *L. erythrorhizon* root exist in the extract from the outer surface, and researchers basically agree that most materials extracted from *L. erythrorhizon* have hydrophobic characteristics and can be efficiently extracted in an organic solvent (*16*). **Table 1** compares the pigment yield and Hunter values of the pigments extracted using various solvents. Methanol and acetone were found to be more appropriate solvents for the pigment extraction than the other solvents used in the extraction. The pigment quantity for each solvent did not continuously increase in proportion to the extraction time and agitation strength. The extract was a mixture of red pigments, and the λ_{max} of the pigment was 520 nm using PDA spectroscopy (data not shown). The *a* value, indicating the degree of redness among the Hunter values, for the pigment in water was the lowest compared to the values in the other solvents used in the extraction, proving that the red pigments in *L. erythrorhizon* were nearly insoluble in water. As such, this confirms the efficacy of using hydrolytic enzymes in aqueous media in the extraction process without a severe loss of the red pigments. Moreover, there were high contents of polar materials except for the pigments in the constituents of the solvent extracts. Thus, hydrolytic enzymes could also be helpful to remove these polar materials that could potentially interrupt and reduce the extractability of the pigment from the tissue. Furthermore, enzymatic pretreatment could increase the dye content in the red-dye production by removing the polar byproducts, thereby increasing the pigment yield from the *L. erythrorhizon* root extraction process.

Measurement of Mass Transfer Coefficient and Pigment Yield. A common approach to enhancing the solute-to-liquid mass transfer is to increase the agitation. The resulting increase in the average shear rate enhances the solvent fluidity, extending the interfacial area for mass transfer. Although effective, this approach is costly for large volume extraction, as the power consumption is proportional to the impeller rate to the third power and impeller diameter to the fifth power (*8*). Most of the pigment ingredients in the *L. erythrorhizon* root are in the outer surface, and this epidermal tissue is mostly composed of a polysaccharide matrix that is strong and packed with several types of saccharide. In this case, agitation is limited with regard to increasing the interfacial area for mass transfer, as the solvent cannot spread between the solid epidermal layer matrices, which means the pigments in the tissue do not come into contact with the solvent. In addition, this compacted structure disrupts the mobility of the pigments in the tissue, which also makes it hard for the pigment as a solute to connect with the solvent. As such, hydrolytic enzymes can solve these problems by destroying the
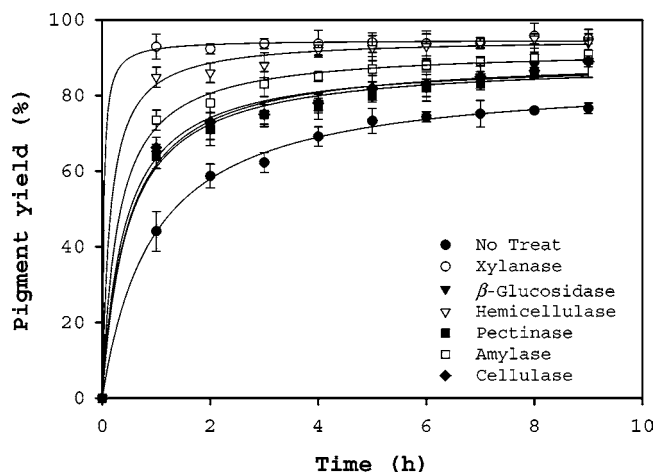
solid structures, because hydrolytic enzymes degrade glycans via the hydrolysis of glycosidic bonds and a β-elimination process between sugars (*17−19*). Thus, in the present study, the outer surface of the root seemed to be loosened with the use of hydrolytic enzymes that disjointed the linkages between the polysaccharides.

The contribution of the enzymes in the extraction process was examined by calculating the mass transfer coefficient, as shown in **Figure 1**, where a good agreement was found between the experimental data and the linear trend predicted by eq 2. The mass transfer coefficient (K_L) was calculated from eq 3 when using various hydrolytic enzymes, and the pigment yield was quantified in the methanol extract after the root-shell tissue had been macerated by each enzyme, respectively. **Figure 2** shows that the pigment yield increased in proportion to the extraction time. Xylanase was superior to the other enzymes, exhibiting a high efficiency in pigment production. It took ∼15 min to obtain 80% of the pigment yield with the xylanase-treated extraction, whereas it took >10 h with the normal extraction without any enzyme treatment. Moreover, the pigments were extracted more rapidly from the enzyme-treated samples, regardless of the kind of hydrolytic enzyme used, indicating that the use of hydrolytic enzymes assisted the pigment extraction by dismantling the epidermal layer of the root. The pigment yield was also generally improved by the enzymes in proportion to the increase in the K_L value calculated in eq 3, suggesting that the increase in the K_L value proportionally agreed with the increment of pigment mobility in the epidermal layer of the root. Therefore, the degree of plant tissue maceration was measured to determine the relationship between the K_L value and the pigment yield after enzyme treatment.

Root Tissue Maceration and Pigment Yield. Although hydrolytic enzymes can release or loosen the tissue structure by dismantling the frame structure, they cannot completely destroy the structure because of their substrate specificities. Therefore, the degree of maceration may help to explain the intensity of the driving force behind the pigment mobility in the structure.

Owing to the hydrolytic properties of the enzymes, 3−14% of the outer surface of the root was disintegrated (**Table 2**). The K_L value for the pigments without enzyme treatment was much lower than that for the samples using the hydrolytic enzymes. Xylanase increased the K_L value of the sample by >2-fold compared to that of the sample without enzyme treatment. The mass transfer coefficient also increased considerably as the degree of tissue maceration increased. Due to the

Table 2. Mass Transfer Coefficient and Pigment Yield after Maceration with Different Enzymes

	tissue maceration[a] (mg)	K_L (mm/s) $\times 10^4$	pigment yield[b] (%)
no treatment		4.534	43.4 ± 2.3
β-glucosidase	3.09 ± 1.59	5.554	64.6 ± 8.1
pectinase	4.05 ± 1.92	6.239	73.5 ± 6.8
hemicellulase	5.30 ± 4.35	5.875	84.8 ± 7.9
amylase	5.31 ± 2.66	7.150	63.9 ± 7.3
cellulase	8.11 ± 1.65	6.453	66.2 ± 9.6
xylanase	13.83 ± 1.76	9.875	92.9 ± 4.8

[a] Results are expressed as means ± SD. Tissue maceration was calculated on the basis of weight loss from each 100 mg sample. [b] Pigment yield was measured from aliquots after 1 h extraction by spectrophotometry. Means ± SD.
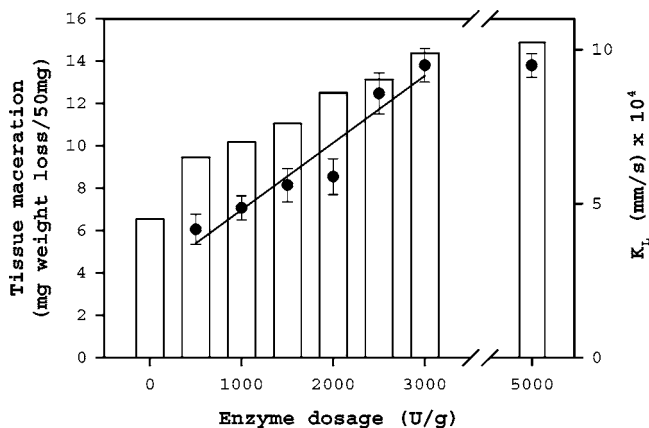

Figure 3. Relationship between K_L, mass transfer coefficient (black dot), and tissue maceration with xylanase (white bar).

pigment mobility, represented by the K_L value, the pigment yield using xylanase was the highest among the samples. Therefore, the disruption of the outer surface polysaccharide network by enzymatic hydrolysis seemingly increased the extract producibility, indicating that the K_L value was related to the velocity of the pigments and permeation of the organic solvent in the shell tissue matrix. This also demonstrated that the degree of tissue maceration reflected the K_L value and pigment yield.

The correlation between the pigment extractability and the mass transfer coefficient was also evaluated according to the hydrolytic activity of xylanase (**Figure 3**). A proportional interrelationship was observed between the degree of maceration and the K_L value up to 3000 units/g of the enzyme; however, above this level the relationship could no longer be maintained, indicating that the enzymatic maceration proportionally increased the interfacial area between the pigment and the solvent.

The reflectance spectra of the pigments were compared to examine the differences in the pigment contents and colors as a result of the enzymatic maceration. As shown in **Figure 4**, almost no differences were found in the reflectance between the pigments produced with or without xylanase in extracts using hexane, acetone, and methanol as the solvent, thereby confirming the possibility of producing pigments with the use of xylanase with little difference in the resulting color.

Natural dye production is traditionally an energy-consuming and water-polluting industry that uses hot water for efficient extraction and tons of solvents for repeated extraction. Thus, solvent recovery has been a focus to minimize costs and pollution. However, this paper proposes a different approach involving enzymes for natural dye production, in which macerating enzymes dismantle the polysaccharide linkage into a loosened tissue matrix. In the present attempt at enzymatic
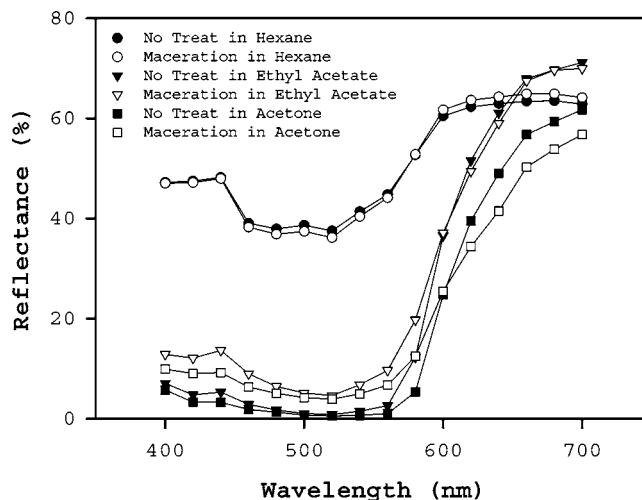
Figure 4. Comparison of reflectance spectra for pigments extracted with/without tissue maceration by xylanase using three solvents.

pigment production, the introduction of macerating enzymes to the initial phase of pigment production was shown to destabilize the epidermal structure in the root shell. Thus, properly conducted maceration can enhance the pigment mobility in the solvent and epidermal structure. Furthermore, the use of hydrolytic enzymes for the maceration enhanced the extractability of the pigment materials. To evaluate the pigment extractability in the solvent resulting from the enzymes, K_L, the mass transfer rate, and degree of maceration were introduced, where K_L represents the pigment flow in the epidermal matrices. The use of the enzymes not only increased the pigment mobility but also sped the process by reducing the time required for pigment extraction. In addition, the plant tissue maceration activity and K_L value with xylanase exhibited a linear proportional relationship. However, xylanase only served to increase the pigment extractability by loosening the shell matrices and had no affect on the coloring characteristics of the natural pigments. Accordingly, it is hoped that this work will extend the field of enzymatic application through the use of xylanase for natural pigment extraction and an increased pigment production yield.

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Development of Enzymatic Pigment Extraction

J. Agric. Food Chem., Vol. 53, No. 7, 2005 **2545**

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