수용성 식물 색소 추출 및 판별에 관한 간편한 방법

Convenient Methods for the Extraction and Discrimination of Water-Soluble Plant Pigments

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요약

식품이나 음료 첨가제로 사용하는 착색제는 소비자가 가공된 식품을 믿고 인정할 수 있는 중요한 요인의 하나로 꼽을 수 있다. 예전에 식품에 안전하다고 다량 사용되었던 인공 착색제나 염료들의 종류는 근래 독성학적 연구 결과 유해하다고 판정되어 그 수가 급격히 줄고 있다. 따라서 안토시아닌이나 베타시아닌과 같이 안전한 천연 색소는 항암성과 항산화성을 갖고 있기에 그 수요가 꾸준히 늘고 있다. 본 연구에서는 수용성 식물 색소인 안토시아닌과 베타시아닌을 에틸 아세테이트, 에틸 에테르 및 클로로포름과 같은 몇 가지 유기용매로 간단히 추출할 수 있었다. 또한, 추출한 이 두 가지 주된 식물 색소는 citrate buffer (pH 3.0)를 이용한 아가로즈 겔 전기영동법 하나만으로 간편하고 빠른 시간 내에 전개하여 서로를 구분할 수 있었다.

■ 중심어 : | 색소 추출 | 안토시아닌과 베타시아닌의 판독 | 겔 전기영동 |

Abstract

The use of colorants as additives for foods and drinks is a significant factor to food consumers in determining the acceptability of processed foods. In recent years, the number of previously used artificial colorants/dyes suitable for food use has been drastically reduced as a result of toxicological studies. Therefore, the use of natural pigments such as anthocyanins and betacyanins that were known to have anticancer and antioxidant activities is increasingly required. In this study, the water-soluble plant pigments, anthocyanins and betacyanins, were easily extracted with a very simple method using a few organic solvents such as ethyl acetate, ethyl ether, and chloroform. After the extraction of them, these two major plant pigments could be also simply and rapidly separated and discriminated by a solely one-stepped agarose gel electrophoresis in a citrate buffer (pH 3.0).

■ keyword : | Pigment Extraction | Discrimination between Anthocyansins and Betacyanins | Gel Electrophoresis |

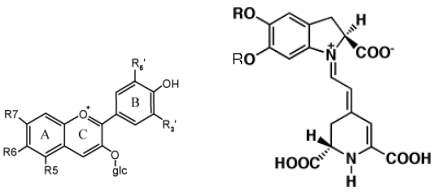
1. Introduction

Color is one of the most important factors determining the consumer's acceptance of manufactured products [1]. Moreover, the use of

접수번호 : #090121-004
접수일자 : 2009년 01월 21일
심사완료일 : 2009년 03월 12일
교신저자 : 정상호, e-mail : shchung@mokwon.ac.kr

proper colorants as additives for foods and drinks is also a significant factor to food consumer and manufacturer alike in determining the acceptability of processed foods. Since the number of previously used artificial colorants/dyes suitable for food use has been drastically reduced as a result of toxicological studies, the use of natural pigments such as anthocyanins and betacyanins [Figure 1] that were known to have anticancer and antioxidant activities is increasingly required. Therefore, these two major classes of plant pigments are concerned in this study. They exist in many plant organs such as flowers, fruits, seeds, leaves, stems, and roots in most of plant species. However, they have never been found together in the same plant and intermolecular co-pigmentation phenomena have not been reported so far [2][3]. Their structural difference is based on divergent biochemical pathways on the arogenate level, the precursor of tyrosine (betalains) and phenylalanine (anthocyanins), respectively [7].

Anthocyanins ([Figure 1]A), a class of flavonoids derived from phenylalanine, are water-soluble, synthesized in the cytosol, and localized in vacuoles [4]. The colors of the charged anthocyanin pigments are dependent on the pH of the intracellular medium containing these pigments. They provide a wide range of colors ranging from orange/red to violet/blue. Their specific color depends on structures, co-pigments, metal ions and pH. Anthocyanins are widely used as natural food colorants. Six major anthocyanidins, aglycons of anthocyanins, are known: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. Their color greatly depends on the number of hydroxyl groups on the B-ring; the larger the number of groups, the bluer the color. Glycosylation of anthocyanins results in slight reddening. The glycosyl moieties of anthocyanins are commonly modified by aromatic and/or aliphatic acyl moieties. Aromatic acylation causes a blue shift and stabilizes anthocyanins. Aliphatic acylation does not change the color but increases the stability and solubility [5].



(A) (B)

Figure 1. Basic structures of anthocyanins (A) and betacyanins (B)

Betalains are alkaloid pigments and are aromatic derivatives synthesized from tyrosine. There are two categories of betalains, betacyanins ([Figure 1]B) and betaxanthins. Betacyanins exhibit the reddish to violet betalain pigments. They are immonium conjugates of betalamic acid with cyclo-dihydroxyphenylalanine (DOPA) glucoside and amino acids or amines, respectively [6]. Betaxanthins are those betalain pigments which appear yellow to orange. Little is known about the role of betalains. They are nitrogen-containing water-soluble compounds that are found only in Caryophyllalles except for Caryophyllaceae and Molluginaceae. The advantage of betalain color is that the color does not depend on the pH and is more stable than that of anthocyanins.

Generally, the procedures of extraction (e.g. via many steps of several solvents treatment, column chromatography, etc.) and discrimination (e.g. via UV-Vis absorption spectrum, HPLC, LC-MS, NMR analysis, etc.) of pigments from plant tissues are tedious, recalcitrant, and time-consuming. The purpose of this investigation is to develop the time-saving simple procedure for the extraction of these two water-soluble pigments as well as for the rapid discrimination between them. In this study,

these two water-soluble plant pigments could be easily extracted with a very simple method using a few organic solvents. These two extracted pigments could also be simply and rapidly separated and discriminated by a solely one-step procedure of agarose gel electrophoresis. The major distinguishing features of this method are the facts that the research time is remarkably shortened and only a few chemicals and equipments are used during the whole experimental procedure.

2. Materials and Methods

Pigment materials

The pigment samples were obtained from the various wild-grown plants or cultivated plants in green house. A great number of pigments were extracted from various plant organ tissues: epidermal tissues of eggplant fruit, grape fruit, sweet potato root, and apple fruit; root of red beet; leaves of red cabbage and *Suaeda japonica*; flowers of cosmos, carnation, rose, chrysanthemum, *Codonopsis lanceolata*, *Lagerstroemia indica, Hibiscus syriacus*, *Mirabilis jalapa*, sage, *Impatiens balsamina*, *Commelina communis,* and *Dendrobium phalaenopsis*; seed coats of black rice, soybean, and azuki bean; fruit of *Schizandra chinensis*. They were washed in distilled water, blotted onto paper towels, and immediately used for pigment extraction or stored at -70°C until use.

Pigment extraction and analysis

The sample tissue was ground in appropriate volume of water (ca. 0.5 ml per g fresh tissue). The used solvents for extraction were ethyl acetate, ethyl ether, and chloroform. After grinding in mortar with pestle, they were centrifuged at 15,000 rpm for 10 min in microcentrifuge and the supernatant was transferred to the fresh vials. The water-extracted pigment compound was further treated with ethyl acetate, ethyl ether, and chloroform successively to remove other organic substances such as chlorophylls, polysaccharides, lipids, and proteins. Each pigment sample was then loaded after being mixed with 1/5 volume of 30% glycerol and separated on a horizontal slab gel electrophoresis apparatus. Electrophoresis of pigments was carried out in citrate buffer (10 mM citric acid anhydrous, 3 mM sodium citrate dihydrate, pH 3.0) on 3.0% agarose gel at 100 V.

3. Results

All the extracted pigment compounds were water-soluble, but insoluble in ethyl acetate, ethyl ether, and chloroform. These pigments were completely distinguished from the photosynthetic green pigment, chlorophyll, as can be seen in solvents of ethyl acetate or ethyl ether ([Figure 2] A and B).

(A) (B) (C)

Figure 2. Water-extracted purple pigment compound after blending with ethyl acetate (A), ethyl ether (B), and chloroform (C). The green chlorophyll pigment was extracted to organic solvents, while the purple pigment was existed in the water layer.

Using this method, it takes a few minutes (within one hour) for the pigment extraction from one sample tissue. Their extracted pigment amounts were also

shown in Table 1. The extracted pigments displayed a wide range of colors ranging from yellow/red to violet/blue according to sample tissues ([Figure 3], -HCl), but some colors were changed at acidic conditions ([Figure 3], +HCl).

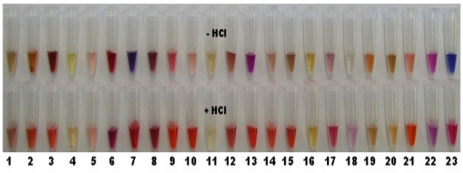


Figure 3. Pigments extracted from various plant tissues.

1, Eggplant; 2, Grape; 3, Sweet potato; 4, Apple; 5, *Schizandra chinensis*; 6, Red beet; 7, Red cabbage; 8, Cosmos; 9, Carnation; 10, Rose; 11, Chrysanthemum; 12, *Codonopsis lanceolata*; 13, *Dendrobium phalaenopsis*; 14, Black rice; 15, Soybean; 16, Azuki bean; 17, *Lagerstroemia indica*; 18, *Hibiscus syriacus*; 19, *Mirabilis jalapa*; 20, Sage; 21, *Impatiens balsamina*; 22, *Suaeda japonica*; 23, *Commelina communis*

Table 1. Pigment amounts extracted from various plant tissues. Each pigment extract was concentrated, dried and weighed. The experiments were repeated two times and each amount presents mean value of them.

Plant species (tissue)	Amount (mg/g fresh weight)
Eggplant (fruit epidermis)	16.9
Grape (fruit epidermis)	29.7
Sweet potato (root epidermis)	40.5
Apple (fruit epidermis)	11.8
Schizandra chinensis (fruit)	115.8
Red beet (root)	46.9
Red cabbage (leaf)	13.7
Cosmos (flower)	13.0
Carnation (flower)	25.1
Rose (flower)	9.7
Chrysanthemum (flower)	23.6
Codonopsis lanceolata (flower)	50.0
Dendrobium phalaenopsis (flower)	25.8
Black rice (seed coat)	122.8
Soybean (seed coat)	99.5
Azuki bean (seed coat)	61.3
Lagerstroemia indica (flower)	11.5
Hibiscus syriacus (flower)	28.0
Mirabilis jalapa (flower)	8.7
Sage (flower)	21.3
Impatiens balsamina (flower)	1.0
Suaeda japonica (leaf)	21.8
Commelina communis (flower)	6.5

However, these water-extracted pigments could not be classified by merely colors whether they belong to anthocyanins or betacyanins. Therefore, gel electrophoretic method was applied to discriminate them. For the clarification, previously well identified and reported anthocyanins and betacyanins were newly selected and extracted from plant tissues and applied to gel electrophoresis as references. The anthocyanin pigments were from red leaves of maple (*Acer palmatum*) [8], and poinsettia (*Euphorbia pulcherrima*) [24], and flowers of begonia (*Begonia semperflorens*) [9], touch-me-not (*Impatiens balsamina*) [12], rose (*Rosa hybrida*) [22], geranium (*Pelargonium* × *hortorum*) [23], morning glory (*Pharbitis nil*) [10], and balloon (*Platycodon grandiflorum*) [11]. On the other hand, the betacyanin pigments were from root of red beet (*Beta vulgaris*) [7], flowers of cockscomb (*Celosia cristata*) [13] and four o'clock (*Mirabilis jalapa*) [14], fruit of pokeweed (*Phytolacca americana*)[15], and red leaf of goosefoot

(*Chenopodium album*) [26]. The gel and buffer concentrations and pH value was critical to this experiment. If the gel concentration is below 3% and the pH value is below 3.0, then gelling problem happens. Besides, if the buffer concentration is higher than that of this method, then the pigment migration will be extraordinarily slow. Usually, the pigment discrimination was possible within a few minutes after running the gel in our method. Consequently, these two kinds of pigments were migrated to opposite directions; the anthocyanins to cathode and the betacyanins to anode, respectively [Figure 4].

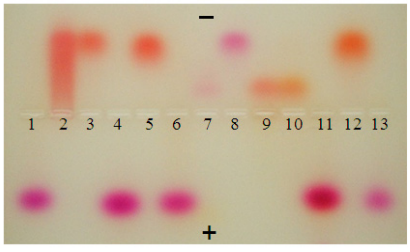


Figure 4. Pigment migration patterns on an agarose gel after electrophoresis. Upper bands, anthocyanins (to cathode); lower bands, betacyanins (to anode).

1, Cockscomb (*Celosia cristata,* betacyanin)
2, Morning glory (*Pharbitis nil,* anthocyanin)
3, Begonia (*Begonia semperflorens,* anthocyanin)
4, Pokeweed (*Phytolacca americana,* betacyanin)
5, Maple (*Acer palmatum*, anthocyanin)
6, Four o'clock (*Mirabilis jalapa,* betacyanin)
7, Touch-me-not (*Impatiens balsamina,* anthocyanin)
8, Balloon flower (*Platycodon grandiflorum,* anthocyanin)
9, Rose (*Rosa hybrida*, anthocyanin)
10, Geranium (*Pelargonium* × *hortorum*, anthocyanin)
11, Red beet (*Beta vulgaris,* betacyanin)
12, Poinsettia (*Euphorbia pulcherrima,* anthocyanin)
13, Goosefoot (*Chenopodium album,* betacyanin)

4. Discussion

According to the report of Jackman and Smith [16], the betalains keep their appearance over the broad pH range from 3 to 7, but degrade at above pH 9. The anthocyanin is not degraded but blue under alkaline conditions. In our study the original colors of the tissues were not changed at all during the extraction procedure, suggesting that the extracted pigments are stable in this method. Generally, anthocyanins display a wide range of colors ranging from orange/red to blue/violet depending on the pH. Their aglycon structures differ in their hydroxylation and methoxylation patterns producing shades from orange/red (pelargonidin) to blue/violet (delphinidin). It seems that the varying ratios of these structures provide a means for plants to achieve all kinds of shades [25]. On the other hand, betacyanins exhibit the red and violet colors. Betacyanins are formed of a betalamic acid unit linked to a molecule of *cyclo*-DOPA. The latter highly aromatic structure is responsible for the deep violet color for the pigment [7][17]. Interestingly, anthocyanins and betalains have never been found together in the same plant so far [2][3][5]. Therefore, the water-extracted pigments in this work must exist in one form of them. However, they could not be discriminated between anthocyanins and betacyanins by merely colors. In the present study, in order to identify the extracted pigments whether they belong to anthocyanins or betacyanins, gel electrophoretic method was applied to separate and discriminate them. At acidic pH 1 to 3, anthocyanins exist chiefly in the form of the flavylium cation [5][18] and occur in the most intense

reddish colors. As the pH increases, deprotonation occurs so that anionic forms of the quinonoidal base predominate at neutral pH [19][20]. On the other hand, the charge carried by betanin, which is a major component of the betacyanins, is influenced by the pH of the solution as follows. The charge of betanin becomes positive with a charge number of one at pH less than 1.5, negative with a charge number of two at pH between 2.5 and 7.5, and a charge number of three at pH between 7.5 and 9.5. The charge of betanin becomes neutral (zero charge) and exhibits an isoelectric point at pH between 1.5 and 2.5 [21]. Using the citrate buffer (pH 3.0) in this study, most of the anthocyanin molecules will be in the positively charged flavylium forms, while the betacyanin molecules will be in the negatively charged forms. With oppositely charged ions they will migrate to opposite directions on agarose gel. Indeed they moved to the opposite directions: anthocyanins to cathode and betalains to anode, respectively [Figure 4]. Therefore, these two pigment molecules could be simply and rapidly discriminated by only one step procedure without using highly complicated analytical equipments.

In this study, the water-soluble plant pigments, anthocyanins and betacyanins, could be easily extracted with a very simple method using a few organic solvents such as ethyl acetate, ethyl ether, and chloroform. After the extraction of them, these two major plant pigments could also be simply and rapidly separated and discriminated by a solely agarose gel electrophoresis in a citrate buffer (pH 3.0). The major characteristic features of our method are as follows: (1) the research time can be remarkably shortened - pigment extraction could be done within 1 hour and pigment discrimination could also be possible within a few minutes after running the gel; (2) a few chemicals are used - three organic solvents for extraction and citrate buffer for discrimination during the whole experimental procedure; (3) only two simple equipments are required - a microcentrifuge and a horizontal slab gel electrophoresis apparatus without any other highly expensive analytical apparatuses such as spectrophotometer, LC-MS, HPLC, and NMR; (4) subsequently, the cost is greatly reduced. Therefore, it seems that these pigment extraction and discrimination methods described herein will be very helpful to apply to another plant species as well as other non-plant organisms such as microorganisms and animals. In addition, theses simple methods will also provide convenience to researchers who are involved in pigments/colorants-related fields.

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