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Stabilization and Color Variation of Anthocyanins with Inorganic Salts

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ABSTRACT

"Umeboshi" is a traditional Japanese pickle colored by anthocyanin from *Perilla ocimoides*; the red color is stable for several years. The real pigments of *Perilla ocimoides* were isolated, and color variation and stability of *Perilla* anthocyanins with inorganic salts were studied. The violet color of anthocyanin in neutral aq. solution was changed to blue with 1 M divalent salts ($MgCl_2$ and $CaCl_2$). Both in acidic and neutral aq. solutions a highly concentrated salt (more than 1 M) stabilized the color of anthocyanin in aq. solution. In particular, 4M $MgCl_2$ exhibits very strong stabilization. The salt prevents hydration of anthocyanin and stabilizes the color. The prevention is caused by lowering water activity with salt. This has been revealed by ^{17}O NMR measurement.

INTRODUCTION

The red, purple and blue colors of flowers, fruits and leaves are mostly anthocyanins. Anthocyanins exist as glycosides in plants and their aglycon, anthocyanidin, is a chromophore of these pigments (Harborne, 1965; Brouillard, 1982; Goto, 1987; Harborne and Grayer, 1988; Goto and Kondo, 1991).

Anthocyanins change their color with pH like litmus (Willstätter and Everest 1913) (Fig. 1). In strongly acidic solutions anthocyanins form flavylium ions and the color is orange to red. In weakly acidic or neutral solutions they first form anhydrobase, whose color is reddish violet to violet. In alkaline media they form anhydrobase anion and show a blue color (Harborne, 1967). Although most physiological pH of plant cell sap is around 6, flower colors have wide varieties. The color variation of anthocyanins in plants cannot be explained only by the pH theory (Shibata et al., 1919) and has thus been one of the major problems in anthocyanin chemistry.

The flavylium ion formed in strong acidic media is very stable. When an acidic solution of anthocyanin is neutralized, the anhydrobase and anhydrobase anion is first formed, but anthocyanin is easily hydrated at the 2-position of anthocyanidin nucleus, then quickly changes to the colorless pseudobase (Brouillard and Delaporte, 1977; Brouillard, 1982) (Fig. 1).

Anthocyanins are contained in many fruits such as apples, grapes, many berries, and vegetables such as red cabbages, red onions, lettuces, and other natural foods, and the pigments exhibit various vivid colors. Therefore, if these two problems could be solved, these pigments could be used widely as a safe food colorant, taking the place of synthetic azo-dyes about which there are doubt concerning hepatic toxicity or carcinogenicity.

In Japan, pigments of leaves of *Perilla ocimoides* (shiso) have long been used for coloring salted pickles, such as pickled plum, "umeboshi", and pickled ginger, "benishoga". It is not known why umeboshi retains such a beautiful red color for several years. Umeboshi is strongly acidic and contains about 20% salt. Salts might therefore be connected with color stability (Goto et al., 1976).

In this study we isolated seven anthocyanins and three flavones from *Perilla ocimoides* and completely determined their structures. The color variations of the major anthocyanin in various inorganic salt solutions and their stabilities were then examined.

MATERIALS AND METHOD

General procedures

Electronic spectra were recorded on a HITACHI UV-228 spectrometer. ^1H NMR (500 MHz) spectra

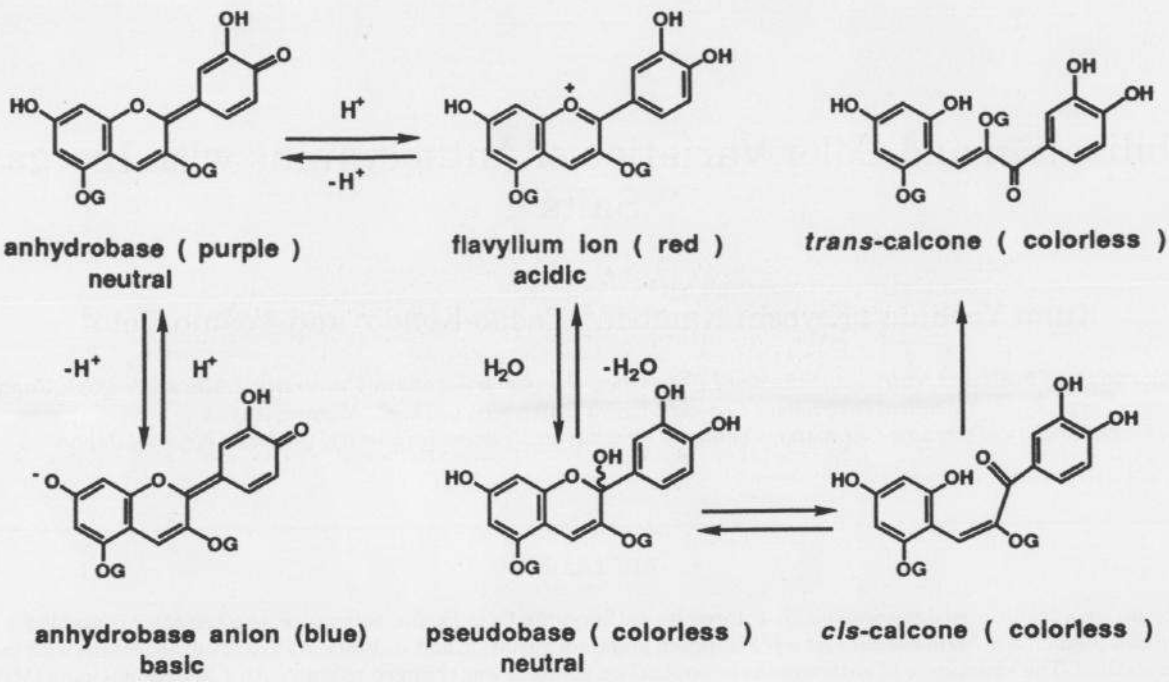


Fig. 1. Structural change of anthocyanin with pH in aqueous solutions.

were obtained on a JEOL GX-500 spectrometer. ^{17}O NMR (36.58 MHz) spectra were measured on a JEOL GX-270 spectrometer. FABMS spectra were recorded on a JEOL DX300/DA5000 and DX304/DA5000 system. HPLC was carried out using JASCO TRIROTAR III equipped with a UVIDEC-III detector and a pair of JASCO 880-PU pumps equipped with a MULTI-340 detector. Inorganic salts and buffer salts were purchased from Hayashi Chemicals.

Pigments

Anthocyanins, flavones and rosmarinic acid were isolated from frozen red shiso leaves (*Perilla ocimoides*) by the method reported by Kondo et al. (1986, 1989), Kondo (1991) and Yoshida et al. (1990) and the purity was checked by HPLC. The structures of each pigments were determined by ^{1}H NMR, FABMS and the degradation method reported by Kondo et al. (1986, 1989), Kondo (1991) and Yoshida et al. (1990).

Electronic spectra and stability of shisonin

To the various pHs buffer solutions (0.1 M) or buffer solutions containing inorganic salts (0–4 M), a solution of shisonin TFA salts in aq. 50% CH_3CN was added, then the pH was adjusted by addition of a small amount of aq. HCl or aq. NaOH. The electronic spectra were measured within 2 min after dissolving the pigment. The solutions were kept at 20 or 80°C and the change of the absorption maximum at visible region were recorded.

^{17}O NMR measurements

Various inorganic salts were dissolved in distilled water. Each sample was measured in 10 mm ∅ tube at 40°C without duetrium lock. The spectral width was 10,000 Hz and a 25 μs pulse was used. The acquisition time and the pulse interval were 0.026 and 0.1 s, respectively. Proton decoupling was used during the measurement time and the times of accumulations were 2,500.

RESULTS AND DISCUSSION

Isolation and structural determination of anthocyanins from *Perilla* leaves

The pigment of *Perilla ocimoides* was first isolated by Kuroda and Wada (1935) and named shisonin. The structure of shisonin was reported by Takeda and Hayashi (1964) and Watanabe et al. (1966) independently as cyanidin 3-(6-*O*-*p*-coumarylglucosido)-5-glucoside. In 1983 Goto et al. isolated a malonyl anthocyanin, malonylawobanin, from *Commelina communis*, although Harborne (1964) had denied the existence of malonyl groups in anthocyanins. In order to extract anthocyanin pigments from the plant body, HCl–MeOH has been used in general, and by this method demalonation and deglycosylation very often occur. We therefore re-examined the structure of anthocyanins from *Perilla* leaves.

Fresh leaves of *Perilla ocimoides* var. *crispa* Benth were frozen in liquid nitrogen, pulverized, and

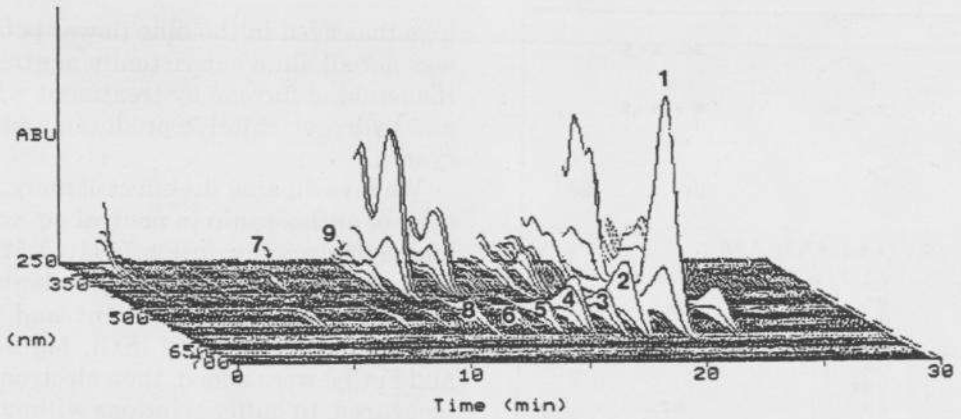


Fig. 2. Chromatograph of the extract from *Perilla ocimoides* using 3% TFA, as detected by a photo-diode array. (From Yoshida et al. (1990), with permission and partially improved.)

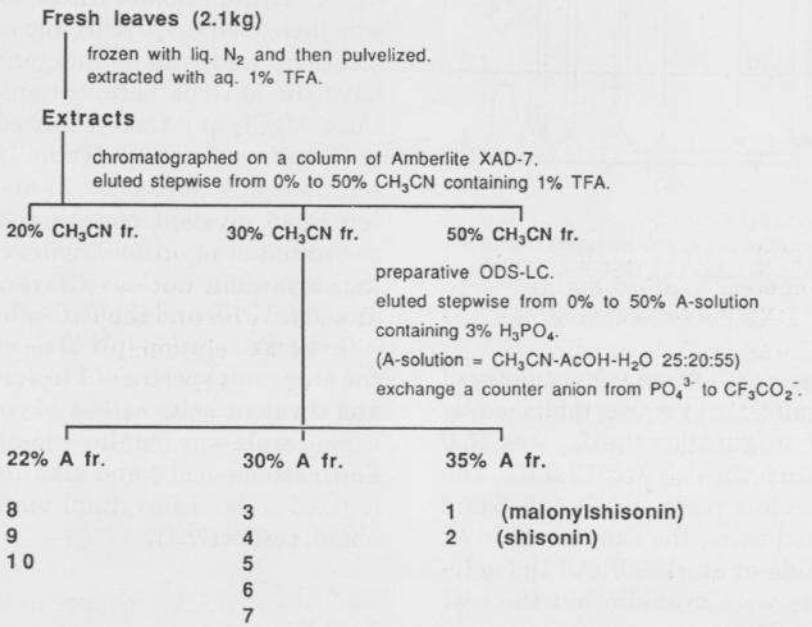


Fig. 3. Isolation procedure of anthocyanins and flavones from *Perilla ocimoides*.

extracted with 3% aq. trifluoroacetic acid (TFA). The extract was immediately analyzed by HPLC of reversed phase column (Asahipak ODP-50) using linear gradient elution from 10% aq. acetonitrile (CH_3CN) to 30% aq. CH_3CN in 30 min. The chromatogram showed the presence of more than ten pigments (Fig. 2). In order to isolate each pigment, large-scale extracts were chromatographed on an Amberlite XAD-7 column and then purified by means of preparative ODS-HPLC (Fig. 3). According to our procedure (Kondo et al., 1986; Goto and Kondo, 1988; Kondo, 1991) seven anthocyanins and three flavones were isolated.

The structure of the pigments was determined completely by FABMS, ^1H NMR (HOMOSD, NOE difference, HOHAHA and ^1H-^1H COSY) and degradation experiments. The structure of the major anthocyanin of *Perilla* leaves was not shisonin but a malonylated anthocyanin, malonylshisonin (1) (Kondo et al., 1989). By FABMS the molecular weight of the major pigment was determined to be 843, while that of shisonin was 757. The difference of 86 mass unit was attributed to malonyl residue, and acidic methanolysis of **1** gave shisonin (2) and methyl malonate. By ^1H-^1H COSY the signals of the two sugars were assigned, then both 6-positions of glucoses are revealed to be acylated (Fig. 4). The positions of glucosidic linkages were determined by NOE

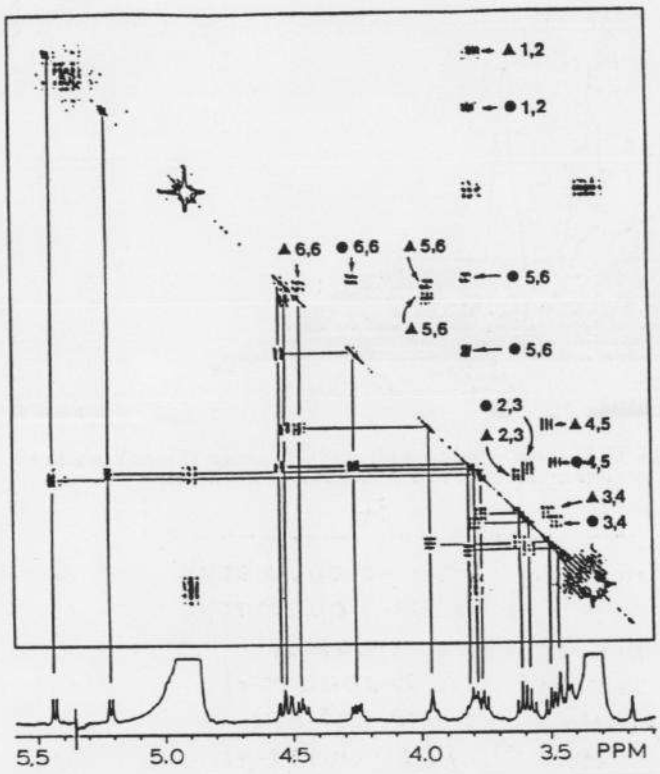


Fig. 4. ^{1}H-^{1}H-COSY spectrum of the sugar moieties of **1** in 3% TFA*d*-CD_3OD at 25°C. (From Kondo et al. (1989) with permission.)

difference spectra. The *cis* (*Z*)-*trans* (*E*) geometry of *p*-coumaric acid and caffeic acid was established by $J_{\alpha,\beta}$ in ^{1}H NMR; in *E* configuration the $J_{\alpha,\beta}$ was 16.0 Hz and in *Z* configuration the $J_{\alpha,\beta}$ was 13.0 Hz. The structures of six other minor pigments (**3**, **4**, **5**, **6** and **7**) were also determined using the same method as shown in Fig. 5 (Yoshida et al., 1990). All the aglycons of these pigments were cyanidin but the acyl moieties were different. Until now *cis*-cinnamic acid derivatives have never been found in natural anthocyanins.

The structure of three flavones (**8**, **9** and **10**) was also determined (Yoshida et al. unpublished) using the above-mentioned method (Fig. 6). From *Perilla* leaves rosmarinic acid (**11**) was isolated (Fig. 7).

Stability and color variation of malonylshisonin in various inorganic salt solutions

(1) Color variation in aq. salt solution

Since the color of anthocyanin changes with the pH of the solution (Fig. 1), Willsttätter and Everest (1913) attributed the variety of flower colors to pH value. Against to the pH theory Shibata et al. (1919) presented the metal-complex theory by their findings that even in the blue flower petals the cell sap was not alkaline but virtually neutral, and that the reduction of flavone by treatment with magnesium and hydrogen chloride produced a blue color anthocyanin.

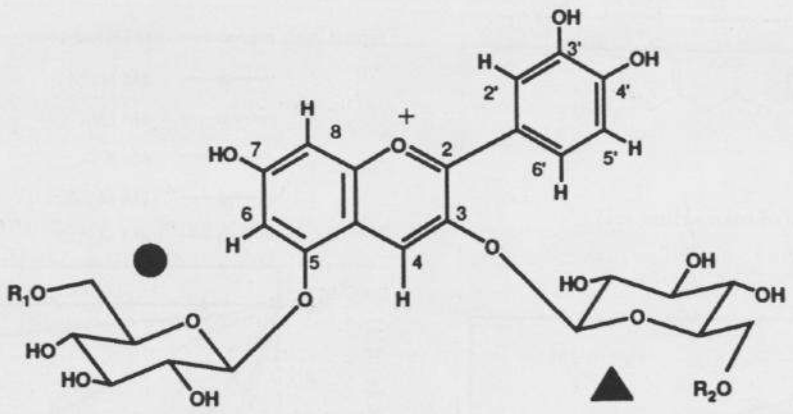
We investigated the effect of inorganic salts to the color of anthocyanin in neutral aq. solutions. To the malonylshisonin solution (5×10^{-5} M) in phosphate buffer (0.1 M, pH 6), 100 equivalents (5×10^{-3} M) of various monovalent, divalent and trivalent inorganic salts (LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $AlCl_3$ and $FeCl_3$) were added, then electronic spectra were measured. In buffer solutions without salt **1** showed purple color and the absorption maximum (λ_{max}) at the visible region was 542 nm. As shown in Table 1 most inorganic salts except $FeCl_3$ had no effect. Addition of $FeCl_3$ gave a bathochromic shift of 7 nm at λ_{vismax}. By addition of the salts the absorbance of **1** was increased (hyperchromic effect). In 1 M aq. salt solutions monovalent inorganic salts still did not have the obvious bathochromic effect but divalent salts, $MgCl_2$ and $CaCl_2$, shifted the λ_{vismax} about 40 nm longer wavelength (Table 1). The color of the salt solutions of **1** were blue. In neutral solutions a concentrated divalent metal ion could presumably be co-ordinated to ortho-dihydroxyl group of B-ring of anthocyanidin nucleus (Bayer, 1966; Hayashi and Takeda, 1970) and the bathochromic shift occurs.

In acidic solution (pH 2) no effect was observed on the electronic spectra of **1** in spite of addition of mono and divalent salts at 1–4 M concentration, and the same result was obtained in alkaline solutions (pH 8). In strong acidic and alkaline solution forms of **1** is fixed to be a flavylium ion and an anhydrobase anion, respectively.

TABLE 1

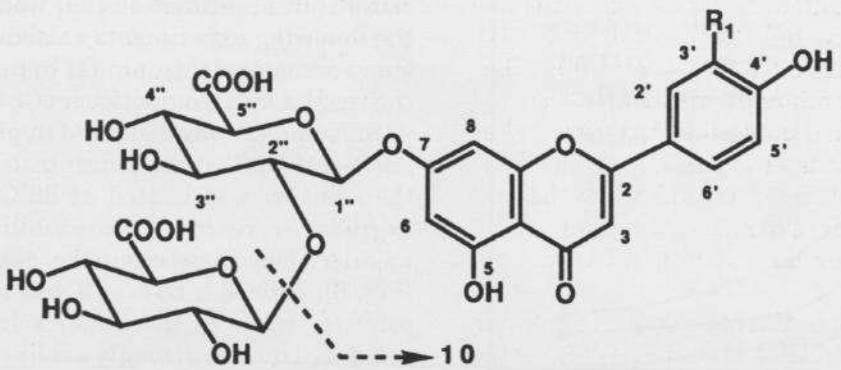
λ_{vismax} (nm) of malonylshisonin (**1**, 5×10^{-5} M) in various salt solutions at pH 6

		Conc. of salt	
		100 eq. (5×10^{-3} M)	1 M
Buffer	542	–	–
NaCl	–	542	548
LiCl	–	542	543
KCl	–	542	546
$MgCl_2$	–	541	581
$CaCl_2$	–	542	583
$FeCl_3$	–	549	–
$AlCl_3$	–	543	–



		R_1	R_2
malonylshisonin	(1)	malonyl	*trans*-*p*-coumaryl
shisonin	(2)	H	*trans*-*p*-coumaryl
caffeylmalonylcyanin	(3)	malonyl	*trans*-caffeyl
malonyl-*cis*-shisonin	(4)	malonyl	*cis*-*p*-coumaryl
caffeylcyanin	(5)	H	*trans*-caffeyl
cis-shisonin	(6)	H	*cis*-*p*-coumaryl
cyanin	(7)	H	H

Fig. 5. Structure of anthocyanins from *Perilla ocimoides.*



		R_1
7-*O*-diglucuronylapigenin	(8)	H
7-*O*-diglucuronylluteolin	(9)	OH
7-*O*-glucuronylluteolin	(10)	OH

Fig. 6. Structure of flavones from *Perilla ocimoides.*

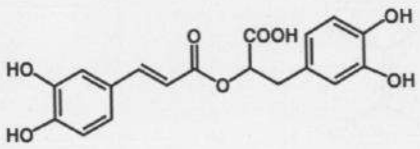


Fig. 7. Structure of rosmarinic acid.

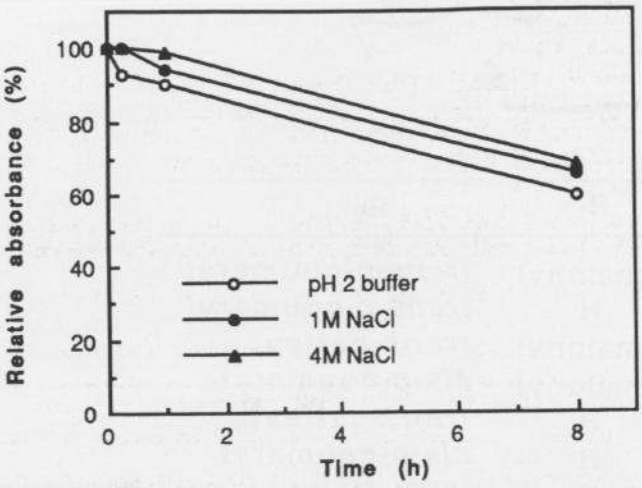


Fig. 8. Color stability of shisonin (2) in NaCl solutions (5×10^{-5} M, pH 2.0, 80°C).

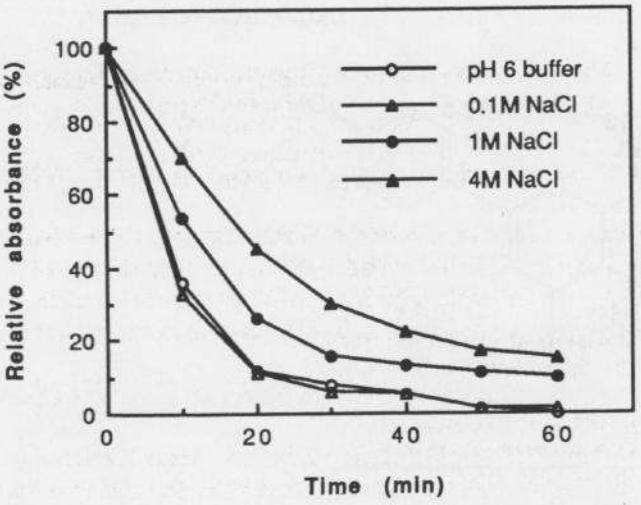


Fig. 9. Color stability of shisonin (2) in NaCl solutions (5×10^{-5} M, pH 6.0, 20°C).

(2) Stability of anthocyanin with inorganic salts

"Umeboshi" is prepared by pickling plums with ca. 20% NaCl by weight accompanying *Perilla* leaves, and retains a beautiful red color for several years. During pickling, a strong acidic liquid composed of various organic acids, umezu, exudes from the plums, so that the umeboshi is under strong acidic condition; ca. pH 1.8. Therefore, the stabilizing effect of NaCl in an acidic solution was studied first. For the following experiments shisonin (2) was used instead of malonylshisonin (1) to prevent confusion of the results by decomposition of 1 to 2.

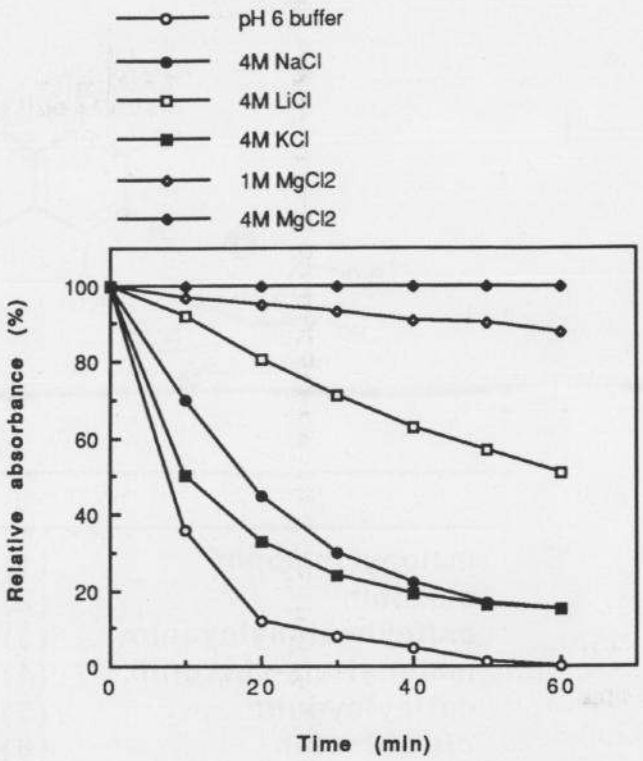


Fig. 10. Color stability of shisonin (2) in 4 M salt solutions (5×10^{-5} M, pH 6.0, 20°C).

Shisonin (2) was dissolved in pH 2 buffer containing 0–4 M NaCl at the concentration of 5×10^{-5} M and the solution was heated at 80°C to accelerate the degradation reaction. The stability of pigment was monitored by measuring the absorbance at λ_{vismax} (Fig. 8). After 8 h 68% of 2 was left in 4 M aq. salt solution, while in the buffer solution 59% of 2 remained. Thus, in strongly acidic conditions, concentrated salts stabilized the red color of the pigment, although to a small extent.

The stability of pigment with salt in neutral aq. solution is shown in Fig. 9. In neutral aq. solution anthocyanins are very unstable and decolorize quickly, so this experiment was carried out at 20°C. Without salt the color of 2 was unstable and became almost colorless within 50 min, but with salt the color of the solution was retained after 60 min. NaCl showed concentrating dependent stabilization.

The stabilizing effect of the other inorganic salts, LiCl, KCl and $MgCl_2$ was also studied (Fig. 10). Shisonin (2) was dissolved in various 4 M salt solu-

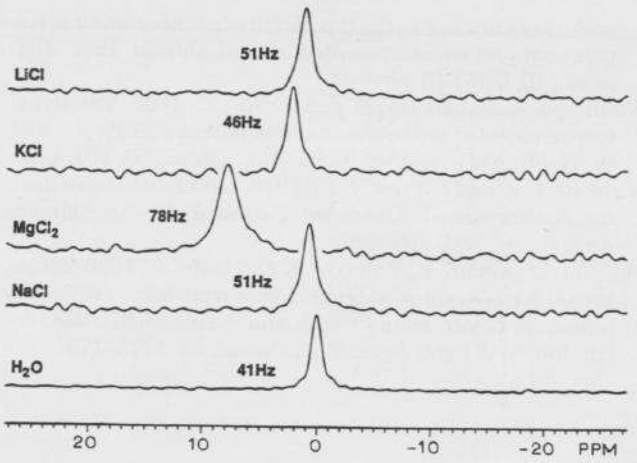


Fig. 11. ^{17}O NMR spectra of H_2O in various 4 M inorganic salt solutions at 40°C (36.58 MHz, ^1H decoupled).

tions at the concentration of 5×10^{-5} M. $MgCl_2$ stabilized 2 considerably and the color remained almost for 60 min; even with 1 M $MgCl_2$ the stabilizing effect was so strong. The blue color was preserved for more than one month in 4 M salt at room temperature. LiCl showed a moderate stabilizing effect. In 1–4 M highly concentrated salt solution all the inorganic salts stabilized the color of shisonin to some extent.

(3) ^{17}O NMR of highly concentrated salt solution

The decolorization of anthocyanin is caused by attacking the 2-position of anthocyanidin nucleus with H_2O molecules. In concentrated salt solutions water activity should be reduced, then the hydration reaction might be prevented. To estimate water mobility ^{17}O NMR has been used (Richardson and Steinberg, 1987). ^{17}O NMR of H_2O in various 4 M salt solutions was measured. The spectra are shown in Fig. 11. A 4 M $MgCl_2$ solution showed the largest line width and 4 M LiCl the next. The line width of ^{17}O signal of salt solutions was correlated to that stabilizing effect, suggesting that suppression of mobility of water molecules with salt leads to stabilization of pigments.

CONCLUSIONS

By addition of various concentrated inorganic salts, anthocyanins are stabilized from hydration and decoloration. Furthermore, with the addition of divalent salt the color of the solution changed to blue. The stabilization mechanism of salts may be caused by suppression of mobility of water molecules and the bathochromic shift may be caused by the chelation of the metal ion to the *o*-dihydroxyl groups of the B-ring of anthocyanidin nucleus. The stability of the red color of "umeboshi" might be caused by the suppression of mobility of water.

ACKNOWLEDGEMENT

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