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Free amino acids and sugars in the flower of *Carthamus tinctorius* L.

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Abstract

Qualitative and quantitative analyses of free amino acids and sugars in the extracts from freshly collected florets of *Carthamus tinctorius* L. were performed by combination of thin-layer chromatography (TLC), automatic amino acid analysis and gas-liquid chromatography (GLC). Sixteen amino acids were detected and their quantitative relations were investigated. Alditol acetate derivatives of free sugars were examined by GLC. The retention time and resolution pattern of the following monosaccharides, rhamnose, arabinose, xylose, mannose and glucose, were ultimately investigated.

Key words: *Carthamus tinctorius L., safflower, amino acid, sugar*

INTRODUCTION

Safflower (*C. tinctorius*) is one of the most useful plant materials which has long been used for a natural dyestuff or a traditional Chinese medicine. Studies by many workers on flowers have been extensively focused on flavonoid chemistry (Kuroda 1930, Mayer and Cook 1943, Wada 1953, Obara and Onodera 1979), and much valuable information is at hand on natural coloring matters. Our investigations led to the expectation that interesting metabolic constituents might be contained in this plant. In a series of studies on the pigment composition of the *C. tinctorius* flower, we have already reported that the plant contains novel chalcones (Takahashi et al. 1982, 1984a, b). The other constituents, such as steroids, terpenoids, sugars or amino acids, however, have been only little known. From ecological and pharmacological view points, a systematic survey

of many unidentified constituents seems to be indispensable for our investigation.

We are at present investigating the qualitative and quantitative contents of free amino acids and sugars in the florets of the sufflower.

MATERIALS AND METHODS

MATERIALS

Safflower was grown in our experimental field. At the full blooming stage, the yellow florets were collected and immediately used as the starting material. Both Amberlite IR-120B and IRA-400 resins were purchased from Organo Chemical Co., Ltd (Tokyo, Japan). All amino acids were obtained from Ajinomoto Co., Inc. (Tokyo, Japan), and were chromatographically pure. Ninhydrin was obtained from Wako Pure Chemical Inds., Ltd. (Osaka, Japan). The following sugars were obtained and were chromatographically pure: mannose from Kanto Chemical Co., Inc. (Tokyo, Japan), arabinose and methyl-β-D-glucoside from Nakarai Chemicals Co., Inc. (Kyoto, Japan), glucose from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), fructose, galactose, lactose, maltose, rhamnose, sucrose and xylose from Wako Pure Chemical Inds., Ltd. (Osaka, Japan). TLC plate for qualitative analysis was carried out on Merck (Darmstadt, West Germany) glass plates precoated with silica gel 60 (0.25 mm) or cellulose (0.1 mm). Developing solvents used for TLC analysis were purchased from commercial sources and all were reagents grade. A Hitachi Model KLA-5 Automatic Amino Acid Analyzer was used for the quantitative analyses of free amino acids. The quantitative experiments for free sugars were carried out on a Shimadzu Gas Chromatograph, Model GC-3BF, with a hydrogen flame ionization detector. The Silicon OV-225 liquid phase and solid support, Chromosob W (AW-DMCS), 80/100 mesh, was purchased from Nippon Kuromato Kogyo Co., Ltd. (Tokyo, Japan).

PREPARATION OF A CRUDE EXTRACT FOR FREE AMINO ACID ANALYSIS

Yellow florets (5.5 kg) were pressed by an instrumental press motor and the resulting juice (3000 cm^3) was concentrated to a volume of 1000 cm^3 by freeze-concentration. Four times the volume of the juice of methanol was added with gentle stirring and the resulting precipitate was removed by centrifugation at 3000 rpm for 5 min. After treating with activated charcoal the supernatant was passed through a column

(3.5 × 70 cm) of Amberlite IR-120B (H^+ form, 500 cm^3) and the adsorbed amino acids were released by eluting with 1 N NH_4OH (4000 cm^3). The eluate was evaporated to a small volume with a rotary evaporator at 40°C and was treated with activated charcoal, then filtered. The filtrate was concentrated in vacuo to give a light brown residue (2.9 g). The residue was dissolved in a minimal volume of distilled water and the solution passed through a column (3.5 × 70 cm) of Amberlite IRA-400 (H^+ form, 300 cm^3). After washing with water, adsorbed amino acids were eluted with 2500 cm^3 of 1 N HCl. The eluate was evaporated at below 40°C, followed by further condensation in a vacuum desiccator over potassium hydroxide. A light brown residue (2.8 g) was obtained.

DETERMINATION OF FREE AMINO ACIDS BY THIN-LAYER CHROMATOGRAPHY

A precoated cellulose plate, 12 cm × 12 cm, was used for the determination of free amino acids. The plates were developed in the ascending fashin in the following solvent systems: A — 1-butanol-acetic acid-water (4: 1: 1, v/v), B — phenol-water (3: 1, w/w), C — 1-propanol-25% ammonia solution (7: 3, v/v), D — ethyl methyl ketone-pyridine-acetic acid-water (70: : 15: 2: 15, v/v). Amino acids were detected on dried TLC plates with the ninhydrin spray reagent. Each spot was then identified by comparing with that of nineteen authentic amino acids.

PREPARATION OF A SAMPLE FOR AUTOMATIC AMINO ACID ANALYZER

An aliquot of the sample (0.8 g) was dissolved in 20 cm^3 of 75% ethanol, which was filtered under suction and the filtrate evaporated at about 60°C. The concentrate was redissolved in 10 cm^3 of the solution for the amino acid analyzer, which was diluted with 50 times of the original volume of the same solution and used for automatic amino acid analysis.

PREPARATION OF A CRUDE EXTRACT FOR FREE SUGAR ANALYSIS

Another sample of yellow florets (50 g) was homogenized in 80 cm^3 of water in a Waring blender and filtered through nylon cloth. After extracting five times with water (80 cm^3), 2400 cm^3 of methanol were added to the combined water extracts and the resulting yellow precipitate was removed by centrifugation at 4000 rpm for 10 min. The supernatant was decolorized by activated charcoal (78.5 mg) and filtered under suction. The filtrate was passed through a column (3.0 × 40 cm) of Amberlite

IR-120B (H^+ form, 50 cm^3), evaporated at 40°C, and dried over silica gel in a vacuum desiccator. The crude sugar extracts were obtained as a brown hydroscopic solid (3.37 g).

DETERMINATION OF FREE SUGARS BY THIN-LAYER CHROMATOGRAPHY

Each 5 mg of seven monosaccharides and three disaccharides (see Table 3) was dissolved in 0.5 cm^3 of water and spotted on a TLC plate. Chromatographies were carried out on cellulose plates in solvent E (1-butanol-ethyl acetate-2-propanol-acetic acid-water, 7:20:12:7:6, v/v) and in solvent F (1-butanol-pyridine-water, 6:4:3, v/v) by being developed twice. A silica gel TLC plate, prepared with 0.1 M sodium hydrogensulfate solution or 0.1 M boric acid solution, was used for sugar separation. The former plate was introduced for using solvent G (ethyl acetate-acetic acid-methanol-water, 12:3:3:2, v/v), and the latter one was used in solvent H (1-butanol-ethyl acetate-1-propanol-acetic acid-water, 7:20:12:7:6, v/v). Sugars were made visible by spraying with the aniline phthalate spray reagent.

GAS-LIQUID CHROMATOGRAPHY OF ALDITOL ACETATE DERIVATIVES OF FREE SUGARS

The alditol acetate derivatives of sugars were obtained following the preparation method described by Kusakabe et al. (1977). A standard of six sugars (each 25 mg: arabinose, xylose, rhamnose, galactose and glucose) and an internal standard, methyl-β-D-glucoside (65 mg), were diluted to 25 cm^3 with water. The ratios of standard sugars/methyl-β-D-glucoside solution were made up as followes: 2.5/1.0, 2.0/1.0, 2.5/2.0, 1.0/2.0, 0.5/2.0 and 0.1/2.0 cm^3. Each sugar sample was reduced with 40 mg of sodium borohydride for 3 h, and then treated at pH 4–5 by adding Amberlite IR-120B (H^+ form). After being filtered off the resins, the filtrate and wash were evaporated 3 times at 40°C by adding methanol. The residue was well-dried in a silica gel desiccator and acetylated with 2 cm^3 of a mixture of acetic anhydride and pyridine. The reaction mixture was then allowed to stand overnight at 30°C. Evaporation of the solvent in vacuo left alditol acetate derivatives. The acetate was dissolved in 2 cm^3 of chloroform and 4 μm^3 subjected to GLC analysis.

Two portions of sample (30 mg) were dissolved in water (3 cm^3) and 0.4 or 2 cm^3 of methyl-β-D-glucoside solution were added. Each sample was then treated in a similar manner as mentioned above.

The alditol acetate derivatives were analyzed on a Shimadzu Model GC-3BF gas chromatograph. The stainless steel column (3 mm × 3 m) used was packed with 2% Silicon OV-225 on 80–100 mesh AW-DMCS

treated Chromosorb W. The injection temperature was 225°C and the alditol acetate was injected at an oven temperature of 180°C. Nitrogen was used as the carrier gas at a flow-rate of 35 $cm^3 \cdot min^{-1}$. Peaks were identified by comparing the relative retention times with standard free sugars. The quantitative ratios of sugars were calculated from the calibration curves based on the methyl-β-D-glucoside peak height.

RESULTS

FREE AMINO ACIDS IN FLORETS

Crude extracts from florets were subjected to preliminary analysis on the analytical cellulose TLC plates. The results which were compared with R_f values of nineteen authentic amino acids are shown in Table 1.

Table 1

R_f values (× 100) of standard amino acids and quantitative determination of free amino acids in florets by TLC analysis using solvents A-D

Amino acid	R_f in solvents [1]				Free amino acids [2] in florets
	A	B	C	D	
Alanine	27	30	36	13	+
Valine	41	38	54	28	++
Leucine	54	47	58	41	+
Isoleucine	51	44	58	37	−
Glycine	22	22	25	08	+
Proline	19	47	28	15	++
Serine	22	18	23	08	++
Threonine	26	26	29	15	++
Hydroxyproline	21	42	19	14	−
Aspartic acid	21	12	11	03	++
Methionine	46	43	57	37	−
Glutamic acid	26	18	13	06	++
Phenylalanine	55	54	57	46	+
Arginine	08	07	13	02	+
Lysine	05	02	12	01	++
Tyrosine	51	46	47	44	−
Histidine	06	17	34	02	+
Tryptophan	60	65	57	54	−
Cystine	08	13	17	02	−

[1] Plate: cellulose. Solvents: A — 1-butanol-acetic acid-water (4: 1: 1), B — phenol-water (3: 1), C — 1-propanol-25% ammonia solution (7: 3), D — ethyl methyl ketone-pyridine-acetic acid-water (70: 15: 2: 15). Spray-reagent: ninhydrin.

[2] The spots were appraised by comparing the intensity of the colored spots which were developed in four different solvents; — not detectable, + weak, ++ strong.

Table 2

The content of free amino acids in safflower florets

Amino acid	Composition	
	μg per 100 g fresh florets [1]	% [2]
Alanine	395	17.4
Valine	58.6	2.58
Leucine	255	11.2
Isoleucine	214	9.40
Glycine	40.8	1.36
Proline	71.1	3.13
Serine	131	5.77
Threonine	34.6	1.52
Hydroxyproline	0	0
Aspartic acid	90.3	3.97
Methionine	11.8	0.52
Glutamic acid	49.6	2.18
Phenylalanine	33.6	1.48
Arginine	90.2	3.97
Lysine	700	30.8
Tyrosine	0	0
Histidine	91.3	4.02
Tryptophan	6.34	0.28
Cystine	0	0

[1] The quantitative relation was determined by an automatic amino acid analyzer.
[2] % of total amino acids obtained.

Seven free amino acids, lysine, aspartic acid, threonine, serine, glutamic acid and valine, were satisfactorily confirmed under different conditions in four solvents. The quantitatively minor amino acids, such as histidine, arginine, glycine, alanine, leucine, phenylalanine, were not fully identified. An automatic method used for quantitative analysis of the free amino acid components in safflower florets is shown in Table 2. The total amino acid content calculated for the fresh florets was 0.23%. The rates of lysine, glycine and leucine were very high compared to other acids components, and their total contents accounted for about 59% of the free amino acids.

FREE SUGARS IN FLORETS

The amino acid-free and water-soluble extracts were separated and analyzed on cellulose or silica gel TLC for sugar composition. The analytical data is given in Table 3. A TLC pattern in solvent F on cellulose was specially resolved. In the safflower florets, glucose, mannose, arabinose

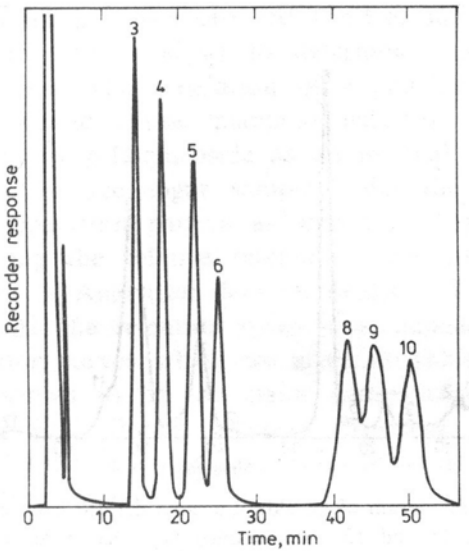

Fig. 1. Gas-liquid chromatogram of an alditol acetate derived from a standard sugar mixture. Peaks: 3 — rhamnose, 4 — arabinose, 5 — xylose, 6 — methyl-β-D-glucoside, 8 — mannose, 9 — galactose, 10 — glucose

Table 3

R_f values (× 100) of standard sugars and qualitative determination of free sugars in florets on different layers using solvents E-H

Sugar	R_f in solvents [1]				Free sugars [4] in florets
	E	F [2]	G [3]	H [3]	
Glucose	22	48	41	21	+
Galactose	20	40	34	16	−
Mannose	29	52	42	23	+
Fructose	29	51	39	19	−
Arabinose	31	53	45	24	+
Xylose	36	60	53	31	−
Rhamnose	51	73	61	44	+
Sucrose	15	42	28	09	−
Maltose	09	34	25	08	−
Lactose	06	26	18	05	−

[1] Plates: cellulose and silica gel. Solvents: E — 1-butanol-ethyl acetate 2-propanol-acetic acid-water (7:20:12:7:6), F — 1-butanol-pyridine-water (6:4:3), G — ethyl acetate-acetic acid-methanol-water (12:3:3:2), H — 1-butanol-ethyl acetate-1-propanol-acetic acid-water (7:20:12:7:6). Spray-reagent: aniline phthalate.

[2] The chromatogram was developed two times with the same solvent.

[3] Prepared with 0.1 M sodium hydrogensulfate solution for solvent G and with 0.1 M boric acid solution for solvent H.

[4] The results were obtained by using four different chromatographic conditions on the plates of both cellulose and silica gel.

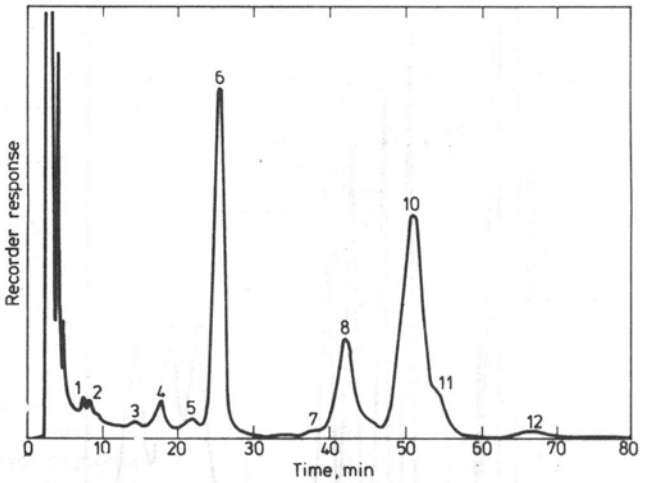

Fig. 2. Gas-liquid chromatogram of an alditol acetate derived from crude extracts of safflower florets. Peaks: 1, 2, 7, 11 and 12 — unidentified, 3 — rhamnose, 4 — arabinose, 5 — xylose, 6 — methyl-β-D-glucoside, 8 — mannose, 10 — glucose

Table 4

The content of free sugars in safflower florets

Peak no [1]	Sugar [2]	Contents	
		mg per 100 g fresh florets	% [3]
1	*	–	–
2	*	–	–
3	rhamnose	t.a. [4]	–
4	arabinose	33.7	2.06
5	xylose	22.5	1.38
6	methyl-β-D-glucoside	–	–
7	*	–	–
8	mannose	422.7	25.9
9	*	–	–
10	glucose	1153.2	70.7
11	*	–	–
12	*	–	–

[1] The manner corresponds to peak numbers shown in Fig. 2.

[2] Each sugar was identified on the basis of retention times of standard sugars, as shown in Fig. 1.

[3] % Of total sugars.

[4] Trace amount.

* Peaks could not be characterized.

and rhamnose were found in flower extracts. The free sugars were analyzed as alditol acetates by GLC analysis to determine the qualitative and quantitative compositions. The separation of a standard mixture of six sugars (rhamnose, arabinose, xylose, mannose, galactose and glucose) was examined by using methyl-β-D-glucoside as an internal standard (Fig. 1). The alditol acetate of a free sugar sample under the same conditions resulted in a good separation pattern as shown in Fig. 2. Peaks were identified by comparing the relative retention times with the authentic chromatograms of Fig. 1. Analytical data for peaks 3, 4, 5, 8 and 10 are in good agreement with the retention times of a standard chromatogram and with the calibration curves, which are given in Table 4. Both glucose and mannose were shown to be the major components at ca. 96% for total free sugars in fresh florets, whereas rhamnose was detected in a negligible amount. Galactose and disaccharides could not be characterized by the present analytical method.

DISCUSSION

In the present study, sixteen free amino acids were identified by applying TLC or an automatic technique for amino acid analysis (see Table 1 or 2). The amino acids detected are familiar to us and moreover all of them are essential in our daily life. The data from chromatographic survey shows that the flower at this flowering period has a relatively normal amino acid composition pattern. In this experiment, no novel amino acid could be found in the flower extracts. The ratio of the amino acid composition varied appreciably according to the individual amino acid contained. The content of the aromatic ring- or sulfur-containing amino acids was remarkably low. On the contrary, it can be seen from Table 2 that the lysine content calculated for the total free amino acid was 0.8%, appearing to be the most prominent. Previously, the high ratio of lysine based on the total amino acids has also been found in the seed hydrolyzates of the same plant (White and Gauger 1967). Its physiological significance in this plant is not fully recognized at this time. The abnormally high content of the basic amino acids, except for histidine and arginine, may reflect the result of some irregular process of amino acid metabolism. Lysine also arose from glucose via the glycolytic pathway. In this process, if a step is restricted by a control mechanism, for example, a route from aspartic acid semialdehyde to homoserine, lysine may accumulate in the plant tissue. However, additional studies are necessary to clarify this interesting problem.

The free sugars in safflower florets were determined by GLC analysis on the basis of the results of a preliminary TLC experiment. The total

sugar content calculated for the fresh florets was 2.01% (see Table 4). This report confirms that in the florets, the sum of glucose and mannose was about 96% of the total sugar content, but no disaccharides were able to be detected. The relatively large amount of glucose found here, coming to 70.7% of the total sugar content, seems to indicate that of it arose being encount from glucosidic compounds. In earlier reports, novel C-glycoside pigments containing glucose have been shown to be contained in this flower. They have intrigued many investigators (Obara and Onodera 1979, Takahashi et al. 1982, 1984a). Mannose belongs to the major sugar components. However, a glycoside pigment binding mannose has not been found in this flower at the present time, Furthermore, mannose has not been identified in other parts of the same plant, safflower kernel and hull (Saunders 1970). We suppose that no mannose is stored by conjugation with the secondary metabolites of the safflower plant.

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Wolne aminokwasy i cukry w kwiecie Carthamus tinctorius L.

Streszczenie

Zrobiono badania jakościowe i ilościowe wolnych aminokwasów i cukrów w ekstraktach ze świeżo zerwanych kwiatków *Carthamus tinctorius* L. za pomocą następujących metod: chromatografii cienkowarstwowej (TLC), automatycznej analizy aminokwasów i chromatografii w ciekłym gazie (GLC). Wykryto szesnaście aminokwasów i zbadano ich relacje ilościowe. Metodą chromatografii w ciekłym gazie określono pochodne octanu alditolu wolnych cukrów. Zbadano też czas trwałości (retention time) i wzór rozkładu (resolution pattern) następujących monosacharydów: ramnozy, arabinozy, ksylozy, mannozy i glikozy.