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Purification and Identification of Guanosine 3′: 5′-Monophosphate from Higher Plants (Evodiae Fructus)

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A cyclic guanosine monophosphate (GMP)-like substance which can be quantitated both by competitive binding assay and by radioimmunoassay has been found in Evodiae Fructus, an oriental herb. This substance has been extracted and extensively purified by Bio-Rad AG 1×4 and aluminum oxide column chromatographies and thin-layer chromatography (TLC). The purified substance was identified as cyclic GMP, since it showed the same physicochemical properties as authentic cyclic GMP on TLC with various solvents, ultraviolet spectroscopy, and high pressure-liquid chromatography and it was found to be decomposable by cyclic nucleotide specific phosphodiesterase.

The existence of cyclic GMP was also demonstrated in several species of Rutaceae. The pharmacological significance of cyclic GMP in this medicinal herb is discussed here.

Keywords——Evodiae Fructus; *Evodia rutaecarpa*; *Evodia officinalis*; Rutaceae; higher plant; guanosine 3′: 5′-monophosphate; cyclic GMP

Introduction

The cyclic nucleotide system has been established as an intracellular second messenger in bacteria and animal cells. However, considerable doubt exists about its occurrence and physiological role in higher plant.[1] Only recently has more evidence been found supporting the existence of adenosine 3′: 5′-monophosphate (cyclic AMP) in higher plants.[2–5] We also reported the presence of large amounts of cyclic AMP in Zizyphi Fructus an oriental herb which has been used for the treatment of asthma.[6,7] Among oriental herbs, many have been described which might have an effect on the digestive function *via* cholinergic stimulation. Thus, it is interesting to test these herbs for the existence of guanosine 3′: 5′-monophosphate (cyclic GMP), which has an antagonistic action on the regulation of cell function in animal tissue.

There are only a few reports describing the cyclic GMP activity in higher plants[8,9] and no report, so far, to confirm its structure. We also found a cyclic GMP-like substance in association with cyclic GMP binding protein (cyclic GMP protein kinase) in Evodiae Fructus.[10] To confirm the existence of cyclic GMP in this medicinal plant, purification and identification of this compound were attempted in this study.

Experimental

Chemicals and Plant Materials——Cyclic GMP, 5′-GMP and cyclic nucleotide-specific phosphodiesterase (EC 1.3.4.17) were supplied by Boehringer–Mannheim GmbH, Germany. Evodiae Fructus, a fruit of *Evodia rutaecarpa* HOOKER *fil. et* THOMSON and *Evodia officinalis* DODE were supplied by Uchida Wakanyaku, Japan, who had imported it from China. Fresh fruits of *Evodia rutecarpa* HOOKER *fil. et* THOMSON were obtained from the Metropolitan Medicinal Plant Garden Tokyo, Japan. After collection, fresh samples were stored in a freezer at −20°C before assay. Dry weights were determined by freeze drying.

Cyclic GMP Assay——Samples were diluted with water and assayed by both competitive binding assay[11] and radioimmunoassay.[12] Kits for these assays were supplied by Boehringer–Mannheim and Yamasa (Japan), respectively.

Column Chromatography——For the purification of cyclic GMP, a column of AG 1×4, (Cl^-) 200—400 mesh (Bio-Rad, USA) was used. Before it was used, the resin was washed in 2.0 M NaOH, water, 1.0 M HCl and finally water again until it was free from Cl^-. A column of aluminum oxide (Alumina Woelm N. Super I,

Woelm Pharma GmbH, Germany) was also used. Fractions were collected at room temperature and the absorbance was measured at 260 nm.

Thin-Layer Chromatography——For identification, a purified sample was subjected to thin-layer chromatography (TLC) in several solvent systems (visualized with the aid of UV light) in comparison with authentic cyclic GMP. The TLC plates used were made of cellulose and silica gel and were supplied by Merck, Germany.

Spectrophotometry——The spectra of the purified sample and authentic cyclic GMP were measured from 200 to 400 nm at room temperature in cuvettes of 1 cm light path with a Hitachi 200-20 spectrophotometer.

High Pressure-Liquid Chromatography——The purified sample was subjected to high pressure-liquid chromatography (Hitachi model 638-30). A column of Hitachi gel 3013N, 4.0 mm × 150 mm was equilibrated with 10% CH_3CN containing 60 mM NH_4Cl, 10 mM K_2HPO_4, and 10 mM KH_2PO_4. Aliquots of the sample were injected onto the column and eluted at a flow rate of 1.0 ml/min at 60°C.

Purification of the Cyclic GMP-like Substance——Extraction: Dried Evodiae Fructus (250 g) was extracted three times with 2.5 l boiling water and the filtered extract glass filter was lyophilized. The residue was refluxed three times with 650 ml of methanol and the filtered extract was concentrated under reduced pressure until completely dry.

Chromatography by Bio-Rad AG 1 × 4 (Large Size): An AG 1 × 4 column, 3 × 25 cm was used to fractionate 100 ml of redissolved extract in water. After the resin had been washed with 1500 ml of water, the cyclic GMP-like substance was eluted by 50 mM HCl and collected in 15 ml fractions. Under these conditions, most of the activity was in fractions 28 to 51. The active fractions were combined. The residue was redissolved in 5 ml of 50 mM ammonium formate and applied to a column of aluminum oxide.

Chromatography on Aluminum Oxide: A column of aluminum oxide, 2 × 15 cm, equilibrated with 50 mM ammonium formate was used. The column was eluted with 50 mM ammonium formate and 5 ml of each fraction was collected. Under these conditions, the cyclic GMP-like substance was eluted in fractions 25 to 74. Active fractions were combined and lyophilized and redissolved in 100 ml of water, then applied to a Bio-Rad AG 1 × 4 column.

Rechromatography on Bio-Rad AG 1 × 4 (Small Column): A small (1.5 × 6 cm) Bio-Rad AG 1 × 4 column was used for further purification. The resin was washed with 150 ml of water, then the cyclic GMP-like substance was eluted with 50 mM HCl and collected in 5 ml fractions. Under these conditions, most of the activity was eluted from fractions 28 to 60. Active fractions were combined as described above.

Thin-Layer Chromatography: The eluate from the small ion exchange column still contained impurities weighing about 15 times more than the cyclic GMP, as calculated from cyclic GMP activity on the assumption of equivalent molecular weight. Thus the concentrated eluate was applied to a TLC plate (cellulose) and developed with *tert*-AmOH–Formic acid–H_2O (3: 2: 1), giving 2 bands with *Rf* 0.30 and 0.20. Of these spots, only that at *Rf* 0.20 had cyclic GMP activity. This spot was cut from the plate and redissolved in water, and the supernatant was lyophilized. Almost 50% of the residue was cyclic GMP as determined from its activity. These results are summarized in Table I.

Table I. Summary of the Purification of Cyclic GMP from Evodiae Fructus

Step	Total dry wt. (g)	Containing cyclic GMP wt. (mg)	Content (%)	Yield (%)
Evodiae Fructus	500	10.2	0.002	100
H_2O extract	130	7.9	0.005	77.7
MeOH extract	70	6.5	0.009	72.2
Large AG 1 × 4 column	4.2	4.0	0.095	44.4
Alumina column	0.44	3.6	0.82	39.8
Small AG 1 × 4 column	0.03	1.8	6.0	20.0
TLC eluate	0.002	1.0	50.0	11.1

Results and Discussion

Identification

For identification, the purified sample was subjected to TLC in several solvent systems. The isolated cyclic GMP-like substance and authentic cyclic GMP had the same chromatographic behavior on TLC in EtOAc–benzene–MeOH (1: 1: 3): *Rf* 0.38 (silica gel), and iso-PrOH–NH_4OH–H_2O (60: 35: 5): and *Rf* 0.6 (silica gel). The sample and authentic cyclic

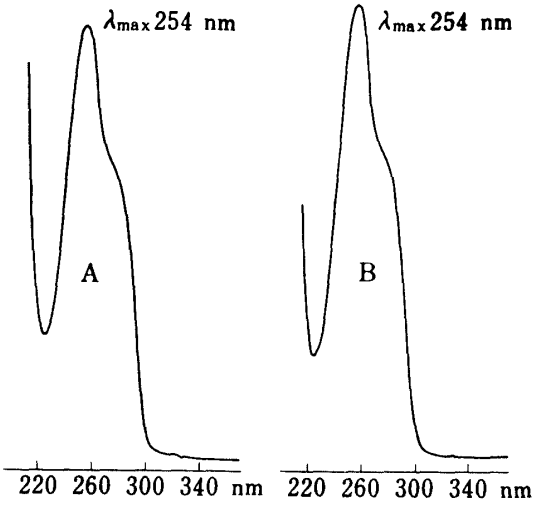

Fig. 1. Ultraviolet Absorption Spectra

Apparatus: Hitachi 200-20 spectrophotometer.
Solvent: water (pH 6.0).
A: purified cyclic GMP-like substance from Evodiae Fructus.
B: authentic cyclic GMP.

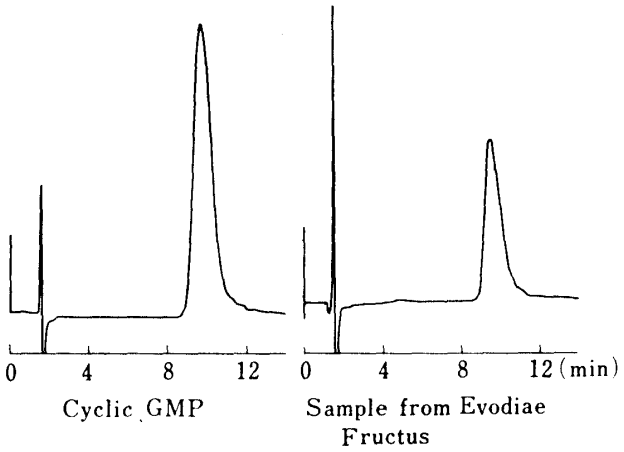

Fig. 2. Patterns of High Pressure Liquid Chromatography

Packing: Hitachi gel 3013N.
Column size: 4.0 mm I.D. × 150 mmL.
Flow rate: 1.0 ml/min.
Temperature: 60°C.
Pressure: 120 kg/cm².
Detector: 254 nm filter.
Eluate: 10% CH_3CN containing 60 mM NH_4Cl, 10 mM KH_2PO_4, 10 mM K_2HPO_4.

GMP were dissolved in water. The maximum UV absorption peak of the purified sample is located at around 254 nm and is indistinguishable from that of authentic cyclic GMP (Fig. 1). On high pressure-liquid chromatography (HPLC), authentic cyclic GMP and the sample gave the same retention time under identical conditions (9.7 min) (Fig. 2). The purified fraction was incubated at 37°C for 30 min with 5.4 mUnits of cyclic nucleotide specific phosphodiesterase in the presence of 5 mM $MgCl_2$ at pH 8.6. The reaction was stopped by heating the mixture to 100°C for 2 min. Almost 98% of the cyclic GMP activity was lost and generation of 5′-GMP was detected by TLC (silica gel) in EtOAc–benzene–MeOH (1 : 1 : 3). These results indicate that the structure of this substance is that of a 3′ : 5′-cyclic monophosphate.

All of these findings unequivocally demonstrate the presence of cyclic GMP in Evodiae Fructus.

Table II. Content of Cyclic GMP of Evodia Genus

Sample	Origin	Fruit	Peduncle
Commercial		nmol/g dry wt.	nmol/g dry wt.
Evodia officinalis Dode	a)	36.8	11.0
	b)	35.0	16.4
Evodia rutaecarpa Hooker *fil. et* Thomson	c)	31.2	27.0
Fresh[d]		nmol/g fresh wt.	nmol/g fresh wt.
Evodia rutaecarpa	21 August	7.7	8.1
Hooker *fil. et* Thomson	1 Sept.	8.4	6.2
	11 Sept.	7.3	6.6

a) Obtained from Tokyo Market (1979).
b) Obtained from Tokyo Market (1975).
c) Obtained from Tokyo Market (1973).
d) Obtained from the Metropolitan Medicinal Plant Garden, Tokyo (1979).

Phytochemical Properties

To ascertain the natural presence of cyclic GMP in Evodiae Fructus and to elucidate the relationship between its pressence and the stage of maturity of the fruit, cyclic GMP activity

was assayed in fresh, intact fruit in various stages of maturity. As shown in Table II, the unripened fruits contained amounts of activity comparable to that found in mature fruits. Also, peduncles and leaves (1st, Sept: 1 nmol/g wet wt.) contained considerable amounts of activity. The existence of cyclic GMP activity was also detected in mature fruits and peduncles of *Evodia officinalis* Dode in amounts roughly equivalent to those found in *Evodia rutaecarpa* Hooker *fil. et* Thomson.

Cyclic GMP activity was detected not only in commercial Evodiae Fructus but also in intact, fresh samples. Even unripened fruits contain activity. These data indicate that an increase in the synthesis of cyclic GMP might have occurred before maturation and the presence of cyclic GMP is not due to bacterial contamination after harvest.

In Chinese traditional medicine, Evodiae Fructus has been used for the treatment of dyspepsia, headaches and microciruculatory disorders. Preliminary experiments done in our laboratory using isotope-labelled cyclic GMP showed that after intra-oral administration of water extract of Evodiae Fructus, cyclic GMP is not absorbed from the intestines. Thus it might act on the gastro-intestinal tract. The normal level of cyclic GMP in the gastro-intestinal tissue is relatively high, but its role in the function of this tissue has not yet been elucidated.[13,14] Comparative studies on the physiological effect of cyclic GMP and the medical use of Evodiae Fructus are currently in progress in our laboratory.

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