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IDENTIFICATION OF GUANOSINE 3′:5′-MONOPHOSPHATE IN THE FRUIT OF *ZIZYPHUS JUJUBA*

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Key Word Index—*Zizyphus jujuba*; Rhamnaceae; guanosine cyclic 3′:5′-monophosphate; cyclic GMP; adenosine cyclic 3′:5′-monophosphate; cyclic AMP.

Abstract—Evidence is presented here confirming the identification of guanosine 3′:5′-monophosphate (c GMP) in the tissue of higher plants. The c GMP activity detected in fruits of *Zizyphus jujuba* was separated from the c AMP activity also present. The separated sample was extensively purified by Bio-Rad AG 1×4 and aluminium oxide CC, and by TLC. The purified sample showed the same physicochemical properties as authentic c GMP by TLC using different solvents and by UV spectroscopy, and was decomposable by cyclic nucleotide-specific phosphodiesterase. The identification was further supported by HPLC. The amount of c GMP present increases 90-fold during fruit ripening.

INTRODUCTION

Recently considerable evidence of the occurrence of adenosine 3′:5′-monophosphate (c AMP) in higher plants suggests the possible participation of c AMP in the action of phytohormones [1–3]. We have also reported the presence of high amounts of c AMP in those higher plants traditionally used as Chinese medicinal herbs [4–6]. There have been only two reports regarding guanosine 3′:5′-monophosphate (c GMP) activity: in tobacco pith tissue using radioimmunoassay on deproteinized tissue extract [7] and in a fraction of bean root partially purified by CC [8]. To date no attempt has been made at purification and identification in plants of c GMP, which has an antagonistic action to c AMP on the regulation of cell function in animal tissues [9]. In the course of tests to determine the presence of c GMP in higher plants, we found c GMP activity in the fruits of *Zizyphus jujuba* and *Evodia rutaecarpa*.

A c GMP sample from *E. rutaecarpa* was partially purified and we suggest it has the same structure as c GMP in animal tissue [10]. However, there were questions regarding the source of c GMP activity in the fruit of *Z. jujuba*, which also contains a great amount of c AMP [4–6]. c AMP cross reacts with c GMP in the assay methods used here, especially in the competitive binding assay where c AMP disturbs the binding of c GMP to the c GMP-binding protein [11]. As a result it may appear that c GMP is present in the samples when it is not.

To confirm the existence of c GMP in the fruit of *Z. jujuba*, we attempted the separation of the c GMP activity from the c AMP activity, and to purify and identify its structure.

RESULTS AND DISCUSSION

The crushed fruit of *Z. jujuba* was extracted with boiling water. The filtered extract was then concentrated and passed through a column of Bio-Rad AG 1×4 (Cl^-) (2×10 cm). The resin was washed with water, both cyclic nucleotides then being eluted with 0.05 N hydrochloric acid. Under these conditions, the c AMP eluted in the early fractions as reported previously [4], whereas c GMP eluted in the later fractions (Fig. 1). This sample, separated from c AMP, was further purified using an Alumina Woelm N. Super I column (2×10 cm), being eluted with 0.05 N ammonium formate.

Fractions containing high c GMP activity were concentrated and further purified by prep. TLC. These results are summarized in Table 1. The isolated

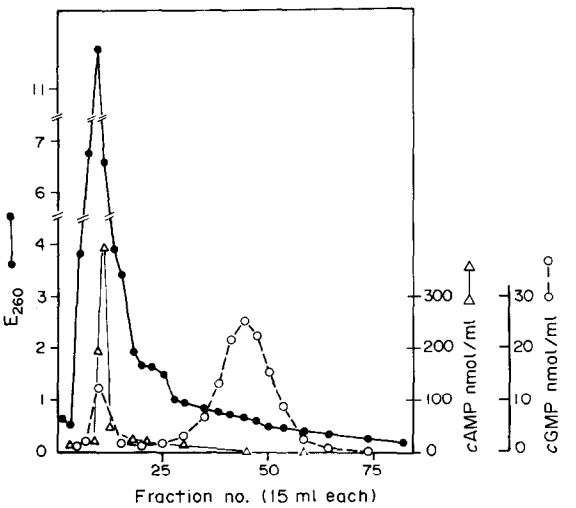

Fig. 1. Elution pattern of c AMP and c GMP using a Bio-Rad AG 1×4 column.

Table 1. Summary of purification of c GMP from the fruit of *Zizyphus jujuba*

Purified step	Dry wt (g)	c GMP content (mg)	Content (%)	Yield (%)
Zizyphi Fructus	100	6.6	0.007	100
H_2O extraction	60	6.0	0.1	91.0
Bio-Rad AG1 × 4 column	0.05	3.5	7.0	53.0
Alumina column	0.012	2.8	23.0	42.4
TLC elute	0.0014	1.5	90.0	22.7

sample and authentic c GMP have the same chromatographic behaviors in TLC in ethyl acetate–benzene–methanol (1:1:3): R_f 0.38 (Si gel), and *iso*-propanol–ammonia–water (60:35:5): R_f 0.6 (Si gel). Both the sample and authentic c GMP were soluble in water. The maximum UV absorption peak of the purified sample is located at *ca.* 254 nm and is indistinguishable from that of authentic c GMP. By HPLC authentic c GMP and the sample gave the same retention times under identical conditions (9.7 min). The purified fraction was incubated at 37° for 30 min with 5.4 munits of cyclic nucleotide-specific phosphodiesterase in the presence of 5 mM magnesium chloride at pH 8.6. The reaction was stopped by heating to 100° for 2 min. Almost 98% of the c GMP activity was lost and generation of 5'-GMP was detected by TLC. These results indicate that the substance is the 3':5'-cyclic monophosphate. To our knowledge, this is the first report confirming the existence of c GMP in higher plants. c GMP levels in the fruit of *Z. jujuba* range from 30 to 60 nmol/g dry wt, as measured by both the competitive binding assay and radioimmunoassay; both methods usually give the same value on a given fruit sample. These values are higher than those in *E. rutaecarpa* [10]. There have been reports of the existence of c GMP-like compounds in higher plants, of amounts up to 100 pmol/g dry wt [7, 8]. The levels of c GMP in the fruits of *Z. jujuba* are therefore the highest yet found, not only in plants but also in animal tissues [12]. The fruit of *Z. jujuba* and *E. rutaecarpa* commercially used in Japan is imported almost entirely from China. Thus the high content of c AMP and c GMP found in the herbs used for the above assays may have originated from contaminating micro-organisms picked up during transportation. As shown in Table 2, however, the data obtained from fresh fruits of *Zizyphus* in Japan indicates that the amount of c GMP in the fruit increases and accumulates considerably during maturation. The amount of c GMP was determined and is shown in Fig. 2 beside that of c AMP. Within 9 weeks, c AMP increased by 35 times and c GMP by 90 times over the original amount. Over-ripe fruit harvested on 11 October shows a decrease in cyclic nucleotides; this correlation detracts from a micro-organism contamination theory, unless these contaminants show a similar growth pattern. Yet home-grown fresh fruits of some other *Zizyphus* spp. also contain c GMP, and screening of some 180 different medicinal herbs, all similarly transported from China, showed that only the fruits of *Z. jujuba* and *E. rutaecarpa* contain c GMP.

In conclusion, the fruit of *Z. jujuba* contains high amounts of both c AMP and c GMP suggesting that

Table 2. Content of c GMP in the fruit of *Zizyphus*

Sample	Date of harvest (origin)	Flesh	Stone
Fresh		nmol/g wet wt	nmol/g wet wt
*Zizyphus vulgaris**	28 July	0.21	0.13
var. *inermis*	12 Sept.	1.6	0
	25 Sept.	16.1	0.23
	5 Oct.	19.3	0.14
*Z. vulgaris**			
var. *spinosus*	12 Sept.	5.4	0
*Hovenia dulcia**		0	0.14
*Malus micromalus**		0	0
Commercial†		nmol/g dry wt	
Zizyphi Fructus (*Zizyphus jujuba*)		30–50	—
			nmol/g dry wt
Z. vulgaris† var. *spinosus*		—	0.41

*Obtained from Takeda Herbal Garden, Kyoto.
†Obtained from Tokyo Market.

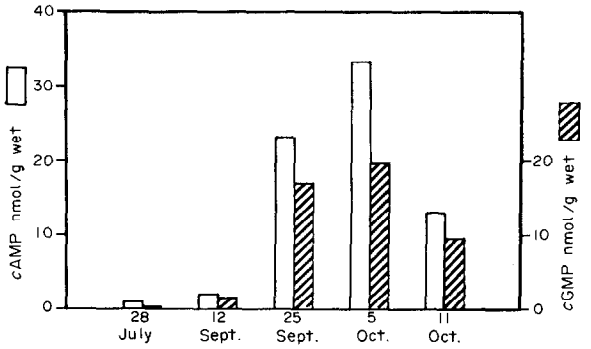

Fig. 2. Change of cyclic nucleotides in the flesh of fruits of *Zizyphus vulgaris var. inermis.*

the specific synthetic mechanism for synthesizing cyclic nucleotides exists in these plants. We are currently studying the possibility that these cyclic nucleotides are the active ingredients in the traditional medicinal recipes from which they were extracted.

EXPERIMENTAL

Plant materials and chemicals. Fruit of *Z. jujuba* and other plants was purchased from Uchida Wakanyaku, Tokyo, who imported it from China. Fresh fruit of *Z. jujuba* was also obtained from Kyoto Takeda Herbal Garden, Kyoto. After collection, fresh samples were stored at −20° before assay. c GMP, 5′-GMP and cyclic nucleotide-specific phosphodiesterase (EC 3.1.4.17) from beef hearts were supplied by Boehringer–Mannheim. Resin of AG 1×4, Cl^- form, 200–400 mesh (Bio-Rad) was used. Before use the resin was washed in MeOH, H_2O, 0.5 M NaOH, H_2O, 0.5 M HCl and finally again in H_2O until it was freed from Cl^-. Also, Alumina Woelm N Super I (Woelm Pharma) was used for CC. The TLC plates used cellulose and Si gel supplied by Merk.

Assay. The sample was diluted with a 50 mM acetate buffer, pH 4 and assayed by both the competitive binding assay [10] and radioimmunoassay [15]. Kits for these assays were purchased from Boehringer–Mannheim and Yamasa, respectively.

Extraction and purification. The crushed fruit of *Z. jujuba* (100 g) was extracted ×2 with 1 l. boiling H_2O the filtered extract was concd *in vacuo*. Repeated boiling H_2O extraction is sufficient to solubilize the c GMP without extracting lipids from the skin of the fruit of *Z. jujuba*, which interfere with our assay. Using boiling H_2O ×10 the weight of the fruit for 15 min; the first extract yielded 29.55, second extract 6.35, third extract 2.97 and fourth extract 0.35 nmol c GMP/g. After four boiling H_2O extractions, a total of 0.22 nmol/g c GMP was obtained by both EtOH and MeOH each twice with ×10 the weight of fruit at 80° for 20 min. Thus, *ca.* 91% of c GMP can be extracted using boiling H_2O (×2). The extract was dissolved in H_2O (60 g/50 ml) and passed through a column of Bio-Rad AG 1×4 (Cl^-), 2× 10 cm. After the resin was washed with 300 ml H_2O, both cyclic nucleotides were eluted with 300 ml 0.05 N HCl. Under these conditions (each fraction, 15 ml) c AMP eluted in the first 7–11 fractions and the c GMP sample eluted in fractions 37–55 (Fig. 1). Active fractions were combined and lyophilized.

The sample separated from c AMP (500 mg) was dissolved in 2 ml 0.05 N $HCOONH_4$, and applied to a column of Alumina Woelm N. Super I (2×10 cm). The sample was fractionated (each fraction, 10 ml) with 300 ml 0.05 N $HCOONH_4$ and fractions 6–12 were concd *in vacuo* (12 mg). Since the concd eluate from the alumina column still contained impurities, further purification was attempted by prep. TLC. The concd eluate applied by TLC (cellulose) and developed with a solvent *t*-AmOH–formic acid–H_2O (3:2:1), giving three bands with R_f 0.80 (I), 0.75 (II) and 0.20 (III). Of these spots, only III had c GMP activity. This spot was cut from the TLC plate, dissolved in H_2O and lyophilized.

Chromatographic comparison. The isolated sample and the authentic c GMP had the same chromatographic behavior on TLC in EtOAc–C_6H_6–MeOH (1:1:3): R_f 0.38 (Si gel), and *iso*-PrOH–NH_4OH–H_2O (60:35:5): R_f 0.6 (Si gel).

UV absorption spectrum. Both the sample and authentic c GMP were dissolved in H_2O at 14 μg/ml.

High pressure-liquid chromatography. The purified sample was subjected to Hitachi HPLC model 638-30. A column of Hitachi gel 3013N, 4×150 mm equilibrated by 10% MeCN soln containing 60 mM NH_4Cl, 10 mM KH_2PO_4 and 10 mM K_2HPO_4. Aliquots of sample eluted under flow rates 1.0 ml/min at 60°. The retention time of the sample was the same as that of authentic c GMP.

Sensitivity to phosphodiesterase. The purified sample (50 pmol/ml) was incubated at 37° for 30 min with 5.4 munits of cyclic nucleotide-specific phosphodiesterase from beef hearts (EC 3.1.4.17) in the presence of 5 mM $MgCl_2$ at pH 8.6. The reaction was stopped by heating to 100° for 2 min. Almost 98% of the c GMP activity was lost and generation of 5′-GMP was detected by TLC; R_f 0.22 (Si gel) in EtOAc–C_6H_6–MeOH (1:1:3). These results indicate that the structure of this substance is probably the same as that of c GMP from animal tissue.

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