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Guanosine 3′: 5′-Monophosphate Activity in Fruits of *Zizyphus jujuba*

JYONG-CHYL CYONG* and MAKOTO TAKAHASHI

Oriental Medicine Research Center of The Kitasato Institute, 5-9-1, Shirokane Minato-ku, Tokyo, 108, Japan

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High levels of cyclic guanosine 3′: 5′-monophosphate (cyclic GMP) activity were detected in the fruit of *Zizyphus jujuba*. A cyclic GMP-like substance was separated from cyclic adenosine 3′: 5′-monophosphate (cyclic AMP), which is also present in the same plant. The levels of cyclic GMP in the fruits of *Zizyphus jujuba* range from 30 to 60 mol/g (dry wt.); these values are the highest yet found, either in plants or in animal tissue. The partially purified cyclic GMP-like substance was identical with authentic cyclic GMP as judged by thin layer chromatography using different solvents and by spectroscopy, and was decomposable by cyclic nucleotide-specific phosphodiesterase.

Keywords——*Zizyphus jujuba*; Rhamnaceae; guanosine cyclic 3′: 5′-monophosphate; cyclic GMP; adenosine cyclic 3′: 5′-monpphosphate; cyclic AMP

We have reported the presence of large amounts of adenosine 3′: 5′-monophosphate (cyclic AMP) in higher plants which have been used as Chinese medicinal herbs.[1–4] We next attempted to find guanosine 3′: 5′-monophosphate (cyclic GMP), which has an antagonistic action to cyclic AMP in the regulation of cell function in animal tissue.[5] In the course of tests to determine the presence of cyclic GMP in higher plants, we found cyclic GMP activity in the fruits of *Zizyphus jujuba* (*Z. jujuba*) and *Evodia rutaecarpa* (*E. rutaecarpa*).

A cyclic GMP-like substance in *E. rutaecarpa* was successfully purified and shown to have the same structure as cyclic GMP from animal tissues.[6,7] However, there were questions regarding the source of cyclic GMP activity in the fruit of *Z. jujuba*, which also contains a large amount of cyclic AMP.[1–4] Cyclic AMP cross-reacts with cyclic GMP in the assay methods used here, especially in the competitive binding assay, where cyclic AMP disturbs the binding of cyclic GMP to cyclic GMP-binding protein.[8,9] As a result, it may appear that cyclic GMP is present in samples when it is not. In order to confirm the existence of cyclic GMP in the fruit of *Z. jujuba*, we attempted to separate the cyclic GMP-like substance from the cyclic AMP.

The crushed fruit of *Z. jujuba* was extracted with boiling water, then the filtered extract was concentrated, and finally extracted with MeOH. The extract was concentrated and passed through a column of Bio-Rad AG 1×4 (Cl^-).

After the resin had been washed with H_2O, both cyclic nucleotides were eluted with 0.05 N HCl. Under these conditions, cyclic AMP was eluted in the early fractions, as reported previously,[3,4] and a cyclic GMP-like substance was eluted in the late fractions. The substance separated from cyclic AMP was further purified on an Alumina Woelm N. Super I

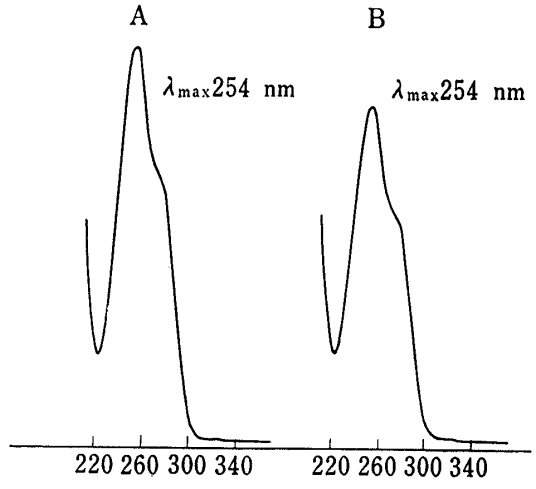

Fig. 1. UV Absorption Spectra

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

column and by thin layer chromatography (TLC).

The purified cyclic GMP-like substance was identical with authentic cyclic GMP as judged by TLC using different solvents, ultraviolet (UV) spectroscopy and high performance liquid chromatography (HPLC).[10] The UV absorption of the sample was indistinguishable from that of authentic cyclic GMP, as shown in Fig. 1. On the basis of this spectrum, the purity of the sample was estimated to be at least 95%. After incubation with cyclic nucleotide-specific phosphodiesterase (EC 3.1.4.17) at 37°C for 30 min, more than 98% of the activity disappeared and 5'-GMP was detected by TLC. These results suggest that the isolated substance is cyclic GMP.

Cyclic GMP levels in the fruit of *Z. jujuba* range from 30 to 60 nmol/g (dry wt.), as measured by both competitive binding assay and radioimmunoassay. Moreover, both methods usually gave the same value for a given sample. These values are higher than those of *E. rutaecarpa*.[6,7] Since boiling water extraction may not have removed cyclic GMP bound to the plant tissue, these values are semi-quantitative and represent a conservative estimate of the concentration of cyclic GMP. There have been a few reports of the existence of cyclic GMP-like compounds in higher plants.[11,12] However, the amounts reported are at most 100 pmol/g (dry wt.). The levels of cyclic GMP in fresh fruit of *Z. jujuba* are therefore the highest yet found, either in plants or in animal tissues.[13] In conclusion, the fruit of *Z. jujuba* contains high amounts of both cyclic AMP and cyclic GMP, suggesting the existence of a specific synthetic mechanism for cyclic nucleotides in these plants.

The relationship between cyclic GMP and cyclic AMP and the maturation of the fruits of these plants is under investigation. We are also studying the role of plant hormones in the synthesis of cyclic AMP and cyclic GMP in *Z. jujuba*.

Experimental

Plant Material——Fruit of *Z. jujuba* and other plants were purchased from Uchida Wakanyaku Company, Tokyo, Japan, who had imported them from China. Fresh fruit of *Z. jujuba* was also obtained from Kyoto Takeda Herbal Garden, Kyoto, Japan.

Assay——Samples were diluted with 50 mM NaOAc buffer, pH 4, and assayed by both competitive binding[9] and radioimmunoassay.[14] Kits for these assays were purchased from Boehringer Mannheim and Yamasa, respectively.

Screening for Cyclic GMP Activity——One hundred and eighty different plants known to Chinese medicine were screened for cyclic GMP activity. Dried plant material (10 g) was supended in 300 ml of H_2O and boiled until the vol. reached 100 ml (*ca.* 40 min). Authentic cyclic GMP was found to be stable during this procedure. The solution was centrifuged at $2500 \times g$ for 30 min and the supernatant passed through a membrane filter. These H_2O extracts were serially diluted with 50 mM NaOAc buffer, pH 4, and cyclic GMP activity was determined by competitive binding assay. Among all the plants tested, cyclic GMP was found only in the fruit of *Z. jujuba* and *E. rutaecarpa*.

Extraction——The crushed fruit of *Z. jujuba* (100 g) was extracted three times with boiling water, then the filtered extract was concentrated *in vacuo*. The residue was extracted three times with MeOH. This extract was concentrated *in vacuo* (60 g/50 ml) and passed through a column of Bio-Rad AG 1×4 (Cl^-), 2×10 cm. After the resin had been washed with 300 ml of H_2O, both cyclic nucleotides were eluted with 300 ml of 0.05 N HCl; 15 ml fractions were collected. Under these conditions, cyclic AMP was eluted in the first 7 to 11 fractions and the cyclic GMP-like substance was eluted in fractions 37 to 55. The substance separated from cyclic AMP (50 mg) was concentrated and dissolved in 2 ml of 0.05 N $HCOONH_4$, and applied to a column of Alumina Woelm N. Super I (2×10 cm). The column was washed with 300 ml of H_2O, then the cyclic GMP-like substance was eluted (each fraction, 10 ml) with 300 ml of 0.05 N $HCOONH_4$. Fractions 6 to 12 were combined and concentrated *in vacuo* (12 mg).

Chromatographic Comparison——The isolated cyclic GMP-like substance and authentic cyclic GMP showed the same chromatographic behavior on TLC in terAmOH–formic acid–H_2O (3:2:1): *Rf* 0.2 (cellulose), EtOAc–benzene–MeOH (1:1:3): *Rf* 0.38 (silica gel), and isoPrOH–NH_4OH–H_2O (60:35:5): *Rf* 0.6 (silica gel).

UV Absorption Spectrum——The sample and authentic cyclic GMP were each dissolved in H_2O at 14 μg/ml. A Hitachi 200-20 spectrophotometer was used.

Sensitivity to Phosphodiesterase——The partially purified fraction (50 pmol/ml) from the Bio-Rad AG 1×4 column was incubated at 37°C for 60 min with 5.4 m Units of cyclic nucleotide-specific phosphodiesterase

from beef herat (EC 3.1.4.17) (Boehringer) in the presence of 5 mM $MgCl_2$ at pH 8.6. The reaction was stopped by heating 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 probably the same as that of cyclic GMP from animal tissues.

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References

1) J. Cyong, K. Hanabusa, and Y. Otsuka, *Proc. Wakanyaku Symp.*, **12**, 1 (1979).
2) J. Cyong and K. Hanabusa, *Phytochemistry*, **19**, 2747 (1980).
3) K. Hanabusa, J. Cyong, and M. Takahashi, *Planta Med.*, **42**, 380 (1981).
4) K. Hanabusa, J. Cyong, M. Takahashi, and S. Hashimoto, *Proc. Wakanyaku Symp.*, **13**, 90 (1980).
5) N.D. Goldberg, R.F. O'Dea, and M.K. Haddox, *Adv. Cyclic Nucleotide Res.*, **3**, 155 (1973).
6) J. Cyong and M. Takahashi, *Phytochemistry* **21**, in press (1982).
7) M. Takahashi, J. Cyong, and K. Hanabusa, *Proc. Wakanyaku Symp.*, **13**, 96 (1980).
8) J. Cyong and H. Okada, *Method in Immunology*, **4**, 1183 (1975).
9) J.F. Kuo and P. Geengard, *J. Biol. Chem.*, **245**, 2493 (1970).
10) J. Cyong and M. Takahashi, *Proc. Wakanvaku Symp.*, **15**, in press (1982).
11) M.K. Haddox, J.H. Stephenson and N.D. Goldberg *Fed. Proc.*, **33**, 552 (1974).
12) C.V. Lundeen, H.N. Wood and A.C. Braun, *Differentiation*, **1**, 255 (1973).
13) A.L. Steiner, D.M. Kipnis, R. Utiger, and C.W. Parker, *Proc. Natl. Acad. Sci.* U.S.A., **64**, 367 (1969).
14) T. Asakawa, T.R. Rusell, and R.-J. Ho, *Biochem. Biophys. Res. Commun.*, **68**, 682 (1976).