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# cAMP PROMOTED PROTEIN PHOSPHORYLATION OF DIALYSED COCONUT MILK

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**Key Word Index**—Coconut milk; adenosine-3′:5′-monophosphate (cAMP); protein phosphorylation.

**Abstract**—cAMP ($10^{-5}-10^{-6}$ M) promoted endogenous protein phosphorylation was carried out with dialysed but not with undialysed coconut milk. Although the undialysed coconut milk contained inhibitors (s) of the cAMP dependent protein kinase(s), the dialysed coconut milk provided the first direct positive effects of cAMP on endogenous protein phosphorylation of a higher plant material.

## INTRODUCTION

Attempts have been made to explore the role of adenosine-3′:5′-monophosphate (cAMP) as a second messenger in higher plants. Although convincing evidence was obtained for the existence of cAMP and related enzymes, sufficient evidence could not be obtained for its physiological role as a second messenger [1]. However, indirect evidence has been found for the presence of cAMP and guanosine-3′:5′-monophosphate (cGMP) dependent protein kinases in higher plants [2–7]. In this paper direct evidence is reported for the occurrence of a cAMP dependent protein kinase and a related inhibitor in coconut milk.

## RESULTS AND DISCUSSION

Protein phosphorylation was carried out with dialysed (DC) and undialysed (C) coconut milk as a source for cAMP dependent protein kinase(s), related inhibitor(s) and endogenous substrate proteins. After phosphorylation, the proteins were seperated by SDS-PAGE, the gels were dried and exposed on appropriate films. The phosphorylation of the proteins, represented in Fig. 1, was carried out with DC, C coconut milk and in mixtures of DC: C of 7:3 and 1:1 with and without of cAMP ($10^{-5}$M).

The protein phosphorylation is stimulated by $10^{-6}$ M cAMP and somewhat stronger by $10^{-5}$ M cAMP for DC against C coconut milk and against the controls O, without cAMP (Fig. 1). The new phosphorylated proteins were in the molecular weight ($M_r$) range of 70 and 60 000.

The weak band in the 70 000 range of the C, O cAMP-lane is not identical with the bands in the DC, $10^{-6}$ and $10^{-5}$ M cAMP-lanes. This was tested by re-electrophoresis and is also apparent in the lane:C, $10^{-5}$ M cAMP. To the best of our knowledge previous demonstrations of the occurrence of cAMP dependant protein kinases in higher plants were indirect. The autoradiography presented here shows directly for the first time, the occurrence of a cAMP-dependent protein kinase activity in a higher plant fruit, and responses of this activity to physiological concentrations of cAMP.

The undialysed coconut milk showed generally, with or or without cAMP, a stronger phosphorylation in the $M_r$ range of 78 000. Absence of added cAMP caused a stimulation of the phosphorylation of protein in the molecular weight range of 27 to 30 000.

The inhibitory effect of the undialysed coconut milk on the demonstrated cAMP dependent phosphorylation is shown by the lanes: C 30%–DC 70%, O cAMP; C 30% DC 70%, $10^{-5}$ M cAMP and C50%–DC 50%, $10^{-5}$ M cAMP. Enhanced mixing ratios from C 30%–DC 70% to C 50%–DC 50% in the presence of cAMP resulted in almost full suppression of the cAMP dependent phosphorylation reactions. First experiments to evaluate the nature of the suppressing substance (s) yielded protein ($M_r$ 9000) from the dialysate. It showed an enhanced inhibitory effect in comparison with its source of undialysed coconut milk.

As described previously, the biological activity of cAMP, extracted from *Zea mays* seedlings [8], could be demonstrated by its ability to stimulate cAMP dependent protein kinase in a membrane fraction from *Drosophila* brain [9]. These attempts were made to demonstrate that a possible *syn–anti* conformation change [10] was not the reason for missing the cAMP dependent protein kinase activity in higher plants.

Although the virgin coconut milk obviously contained an inhibitor, the dialysed coconut milk provided the first direct positive effects of cAMP on the endogenous protein phosphorylation for a higher plant material.

## EXPERIMENTAL

*Plant material and chemicals.* Coconuts were purchased commercially and used as the source for the milk. [$\gamma$-$^{32}$P]-ATP was obtained from Amersham Buchler, Braunschweig, F.R.G. Coomassie Blue R, G 250, bovine serum albumin, guanosine-5′-triphosphate (GTP) and cGMP were purchased from Sigma, München, F.R.G. Acrylamide, *N′,N′*-methylene-bis-acrylamide (BIS), *N′,N′*-tetramethyl-ethylendiamin (TEMED), ammonium-peroxodisulphate (PER), 2-amino-2-(hydroxymethyl)-1,3-

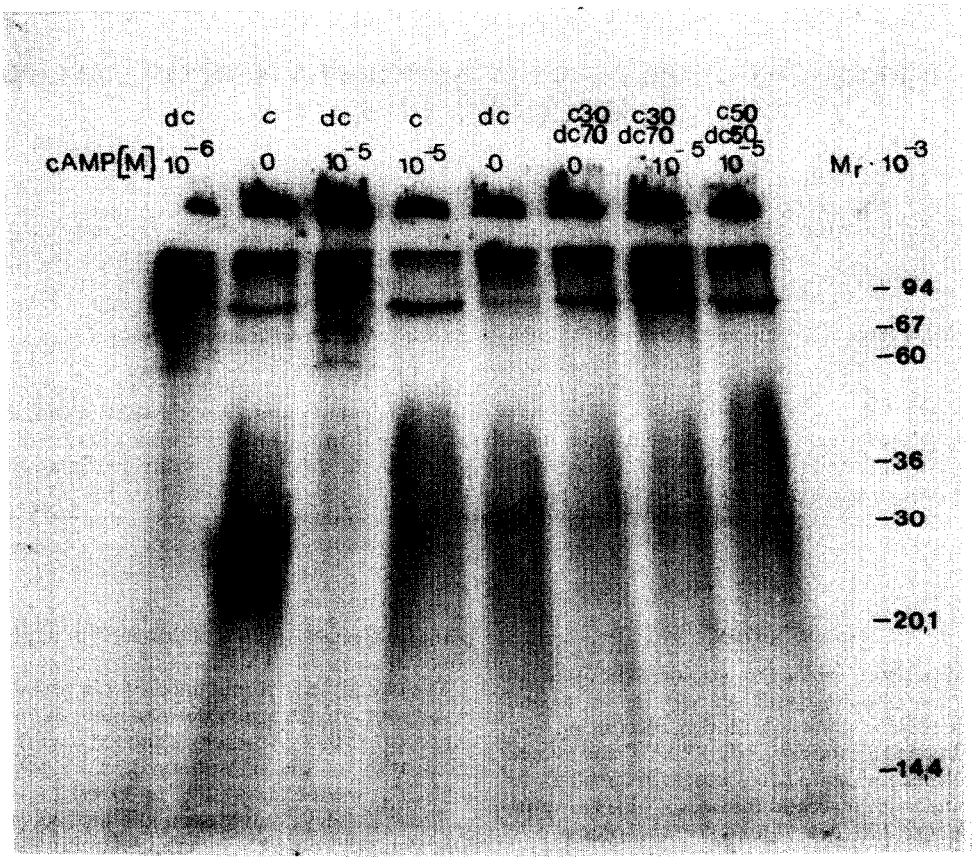


Fig. 1. Authoradiography of $^{32}$P-labelled proteins formed from endolgenous proteins by coconut milk protein kinase activity. From left to right: dialysed (DC) and undialysed (C) coconut milk. Mixing ratios of DC and C: DC 70%–C 30%; DC 50%–C50%. M = molarity of cAMP in the samples. $M_r$ = relative molecular weights. For further information see Results and Discussion.

propanediol (Tris), ethylenediaminetetraacetic acid (EDTA), Mg- and Mn-acetate, adenosine-5'-triphosphate (ATP), cAMP and sodium dodecylsulphate (SDS) were from Merck, Darmstadt, F.R.G. Molecular weight calibration kits were obtained from Pharmacia, Freiburg, F.R.G. Kodak X-Omat AR5 films were from Siemens, Mannheim, F.R.G.

*Dialysis of the coconut milk.* The milk of a coconut (~ 18 ml per coconut) was transfered under sterile conditions into an injection syringe with a membrane filter of 0.22 μm (Millipore). One-half of the filtrate was stored at 4°, the other was placed in a prepared sterile dialysis tube. Before use, the tubes (Thomapor Standard, exclusion limit ~ 10000) were prepared as follows: the tubes were boiled under stirring over 10 min in 1% aq. $Na_2CO_3$, rinsed with $H_2O$ at a pH of 6–7 and stored in an aq. soln of 0.1 mM EDTA at 4°. The dialysis was performed with stirring for 12 hr at 4° against 1 l. of buffer A. The buffer contained: 10 mM Tris–acetate (pH 6,6); 10 mM Mg- and 1 mM Mn-acetate; 0.1 mM ATP; 0, 01 mM GTP and was changed three times: after 12, 8 and 4 hr. The dialysed coconut milk was stored in 40 μl portions at −20° and conttained usually 20 μg protein per portion [11]. The undialysed coconut milk contained 28 μg protein in 40 μl and was therefore stored in 28.5 μl portions (equal protein amounts) at −20°.

*Phosphorylation of the endogenous proteins.* The method used for phosphorylation of the proteins of the both coconut milk samples was as described in ref. [12] with modifications. The standard reaction mixtures of 50 μl contained: 40 μl of the dialysed (DC) or 28.5 μl of the undialysed (C) coconut milk. To the 28,5 μl samples 11, 5 μl 3,5-fold concentrated buffer A was added. For the inhibitor-screening experiments, the DC and C samples were mixed in different ratios. The samples were kept on ice and 5 μl of $H_2O$ or 5 μl of a soln of cAMP were added, reaching the final concentrations of $10^{-6}$–$10^{-5}$M. The reaction was started by the addition of 185 kBq [$\gamma$-$^{32}$P]-ATP in 5 μl $H_2O$ at 30°. The reaction was allowed to proceed for 5 min and it was terminated by transfering the mixtures on ice and addition of 50 μl sample buffer containing 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 63 mM Tris-acetate (pH 6, 8) and a trace of bromophenol blue. The samples were heated for 5 min in a boiling water-bath and 90 μl of each sample was processed for SDS-PAGE.

*SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).* Labelled proteins were separated by SDS-PAGE following the method of Laemmli [13]. The separation gel contained 12, 5% acrylamide and the stacking phase contained 4,5% acrylamide. A 90 μl aliquot of the reaction mixtures, containing usually about 18 μg protein per track, was loaded on the 1 mm thick slab gels and run for 4.5–5 hr at 3 $Wgel^{-1}$. After electrophoresis, when the dye-front had left the gel, the gel was stained for 1–2 hr using O, 25% Coomassie Brilliant Blue in a mixture of 45%

MeOH 10% HOAc and 45% $H_2O$. The gel was destained overnight using a mixture of 5% MeOH, 7% HOAc and 88% $H_2O$. The destained gels were mounted onto a Whatman filter paper and dried using a slab-gel drier. The dried gels were laid on a Kodak X-Omat film and exposed in a cassette at room temp. Exposure time was usually between 48 and 96 hr.

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## REFERENCES

1. Newton, R. P. and Brown, E. G. (1986) in *Receptors in Plants and Slime Moulds* (Chadwick, C. M. and Garrod, D. R., eds), pp. 115–153. Cambridge University Press, Cambridge.
2. Sim, W. S., Roh, K. S. and Moon, H. Y. (1983) *Kor. Biochem. J.* **16**, 27.
3. Kato, R., Uno, J., Ishikawa, T. and Fujii, I. (1983) *Plant Cell Physiol.* **24**, 841.
4. Kato, R., Uno, J., Ishikawa, T. and Fujii, I. (1984) *Plant Cell Physiol.* **25**, 691.
5. Ejiri, S. and Honda, H. (1985) *Biochem. Biophys. Res. Commun.* **128**, 53.
6. Janistyn, B. (1986) *Z. Naturforsch.* **41c**, 579.
7. Janistyn, B. (1988) *Phytochemistry* **27**, 2735.
8. Janistyn, B. (1981) *Z. Naturforsch.* **C36**, 193.
9. Janistyn, B. (1988) *Z. Naturforsch.* (in press).
10. Gronenborn, A. M., Clore, G. M., Blazy, B. and Baudras, A. (1981) *FEBS Letters.* **136**, 160.
11. Spector, T. (1978) *Anal. Biochem.* **86**, 142.
12. Kelly, L. (1981) *Comp. Biochem. Physiol.* **B69**, 61.
13. Laemmli, U. K. (1970) *Nature* **227**, 680.