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IDENTIFICATION OF AN APYRASE ACTIVATING PROTEIN AND OF CALMODULIN IN *SOLANUM TUBEROSUM*

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; calmodulin; apyrase.

Abstract—Three types of effector proteins have been isolated from a partially purified protein preparation of potato tuber. One of the proteins is a typical calmodulin which has no effect on apyrase. The two other proteins modulate ATPase and ADPase activities; one of them with an activating and the other with an inhibitory effect on apyrase. Calmodulin from potato tuber purified to homogeneity had a M_r of 17 500 and an isoelectric point of 4.4. Although the apyrase activating protein is not a pure fraction it differs from calmodulin because unlike this protein it is independent of calcium and does not activate cyclic nucleotide phosphodiesterase from bovine heart. Treatment of the activating protein with tetranitromethane reduces its effect on apyrase, while no change was detected upon treatment with bis-dithionitrobenzoic acid.

INTRODUCTION

Apyrase (EC 3.6.1.5.) is a ubiquitous enzyme that hydrolyses pyrophosphate bonds of organic and inorganic compounds in the presence of bivalent metals. Apyrase activity has been reported in several plant tissues [1–3] and animal tissues [4–6]. Our group has been studying potato apyrase isolated from the tuber of several clonal varieties of *S. tuberosum*. We have found different isoenzymes depending on the variety of potato. These isoapyrases exhibit different kinetic and physicochemical properties [7].

Progress has been made in the field of the structural [8], kinetic and physicochemical properties [7] and of the amino acid residues involved in the active site of apyrase [7, 9, 10], but the physiological role of apyrase is practically unknown. Some authors propose that apyrase could play an active role either in the processing of secretory proteins in the condensing vacuoles or in the exocytosis of the granule contents in the pancreatic cell lumen [4]. Ribeiro *et al.* [6] have found apyrase in salivary gland of blood sucking insects. Due to the central role of ADP in the platelet aggregation, apyrase could hydrolyse ADP preventing haemostasis and facilitating the insect feeding [11]. Vara and Serrano [3] suggest that apyrase could be involved in ion transport in plants as an enzyme bound to membranes because these authors found this enzyme in microsomal fraction of chick-pea roots.

Since there is sufficient knowledge of structural and functional properties of apyrase from potato, it seemed appropriate to explore the regulatory effects on these enzymes. Exploratory experiments *in vitro* with NAD^+, UDPG and other carbohydrate metabolites were consistently negative. A preliminary report of the presence of the regulatory protein calmodulin in *Solanum tuberosum* [12] and the effect of this protein from corn on ATPases in the same species [13] suggested the possibility that apyrase activity could be controlled by regulatory proteins and not by small molecules. The present communication describes two proteins from *S. tuberosum* which modify the activity of apyrase of the same species.

RESULTS AND DISCUSSION

Purification of calmodulin

Calmodulin was purified from potato tuber using fractionation procedures, followed by affinity chromatography. The usual procedure [14] was modified in order to adapt it to the low protein contents of the starting plant material. An ammonium sulphate precipitation must precede the initial heating step in order to increase protein concentration. The partially purified fraction obtained after salt precipitation and heating (Fr-50) behaved as calmodulin since it was able to stimulate bovine heart phosphodiesterase activity in the presence of calcium (Table 1); an effect which could be reversed by trifluoperazine and EGTA. In addition, it stimulated apyrase by 74%. The fraction, Fr-50, obtained by heating a protein concentrate from potato tuber activates both PDE and apyrase to a comparable extent (Table 1). Affinity chromatography of Fr-50 yields three main protein peaks. Two of them eluted with 0.5 M sodium chloride at 0.84 and 2.6 bed volumes respectively, and the third one eluted

Abbreviations: PDE, cyclic nucleotide phosphodiesterase; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; TFP, trifluoperazine; DTNB, bis-dithionitrobenzoic acid; TNM, tetranitromethane; CaM, calmodulin; MES, 2-*N*-morpholino ethanesulphonic acid; TES, *N*-tris-(hydroxymethyl-2-aminoethane sulphonic acid).

Table 1. Effect of protein fraction eluted from TFP-Sepharose column and bovine CaM on PDE and on apyrase

Protein fraction	Column bed volumes	Total protein (mg)	PDE Activity		Apyrase activity	
			Fraction added (μg)	Activation (%)	Fraction added (μg)	Activation or inhibition (%)
Fr-50		21.3	210	98	140	74
Fr-A	0.84	2.2	326	0	22	51
Fr-I	2.6	0.1	15	0	10	−26†
Fr-CaM	0.84	0.2	29	50	20	0
CaM*			3.4	64	3.4	0

PDE and apyrase assay conditions are described in the Experimental section. *S. tuberosum* cv, Ultimus apyrase basal activity was measured, preincubating this enzyme with BSA of the same protein concentration of each fraction tested.

*Bovine heart calmodulin.

†Inhibitory effect.

with 10 mM EGTA. The fraction emerging between 0 and 0.84 bed volumes of 0.5 M sodium chloride exhibits an activating effect only on apyrase, being inactive on PDE. The following peak has no effect on PDE and slightly, but consistently inhibits apyrase. The final peak (Fr-CaM) exhibits calmodulin activity on PDE, but does not affect potato apyrase. Bovine heart calmodulin had no effect on apyrase up to a concentration of 3.4 μg/ml (Table 1). It may be concluded that Fr-50 contains at least three types of effector proteins: A typical calmodulin (CaM) devoid of effect on apyrase, an apyrase activator (Fr-A) and an apyrase inhibitor (Fr-I).

Characterization of calmodulin fraction

Molecular mass. The fraction emerging with 0.84 bed volumes (Fr-CaM) when analysed by polyacrylamide gel electrophoresis in the presence of SDS showed a single protein band of M_r 17 500.

Isoelectric point. Fraction CaM from *S. tuberosum* L. cv Ultimus focused as a single band with a pI of 4.4 which agrees with the values described for plant [15] and mammalian calmodulins [12].

Fraction CaM from potato stimulated phosphodiesterase and had a calcium dependence similar to the activation described for bovine heart calmodulin. As reported for calmodulin from other sources [16, 17] it was inhibited by trifluoperazine (Table 2). The results described, M_r, pI, heat stability and enzyme activator, characteristic of plant [15] and mammalian calmodulin [12], support the conclusion that Fr-CaM from potato is a calmodulin.

Apyrase activator

The protein fraction eluted from the TFP affinity column activated apyrase, but had no effect on PDE. It could be split into several bands by SDS electrophoresis and also by electrofocusing. It is possible that it may be contaminated with the following inhibitory fractions, since the effect of increasing amounts of this activating fraction reached a maximum of about 80% activation and then declined (Fig. 1). The apyrase activator Fr-A stimulated to the same extent the four highly purified iso-

Table 2. Modifications of potato calmodulin effects on PDE activity

Assay mixture	Activation (%)
1. Standard + 3.4 μg bovine heart CaM	60
2. Standard + 20 μg potato CaM	64
3. As 2 + 1 mM EGTA*	28
4. As 2 + 2 mM EGTA*	0
5. As 2 + 50 μM TFP	0

PDE standard assay conditions were: 16 mM cAMP, 30 mM Tris–HCl pH 8, 4 mM Ca^{2+}, 4 mM Mn^{2+} and 100 μg of enzyme.

*No Ca^{2+} was added to the assay mixture.

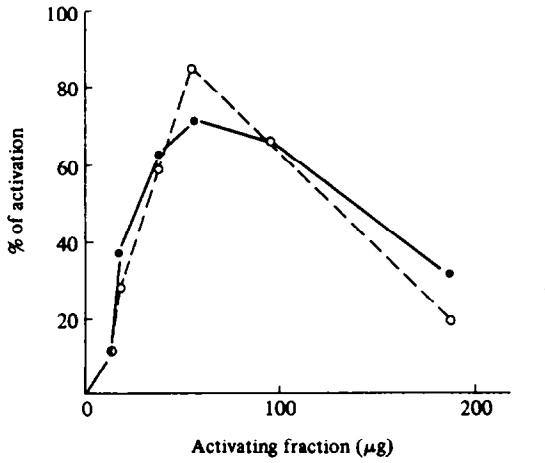

Fig. 1. Protein concentration effect of activating fraction on apyrase activity. The effect of increasing amount of Fr-A on ATPase O---O and ADPase ●——● activities was compared with preincubation with an equivalent BSA concentration. Apyrase assay was performed at pH 6 in 0.1 M sodium succinate, 5 mM $CaCl_2$ in the presence of 2 mM ATP or ADP.

Table 3. Effect of apyrase activator protein (Fr-A) on isoapyrases from different potato cultivars

Isoapyrase	ATPase (%)	ADPase (%)
Desirée + activator	76	59
Pimpernel + activator	68	82
Ultimus A* + activator	75	77
Ultimus B* + activator	75	83
Ultimus A + activator plus 10 mM EGTA	75	80
Desirée + 0.5 mM TFP	16	24

Controls were done by preincubation of each isoenzyme with 40 μg of BSA in 0.1 M NaCl, followed by apyrase assay (ATPase and ADPase), as described in the Experimental section. The amount of activator added to the preincubation mixture of the different isoenzymes was 40 μg of protein.

*cv ultimus apyrase isoenzymes.

apyrases obtained in our laboratory from different varieties of *S. tuberosum* tuber (Table 3).

The addition of 10 mM EGTA did not change the activating effect of this fraction on apyrase but 0.5 mM TFP reduced its effect considerably as shown in Table 3. Since this shows some interaction between TFP and Fr-A, it may explain the binding of the latter to the affinity column. The interaction between apyrase and the activating protein Fr-A was independent of pH in the range 5.0–8.0.

With the purpose of exploring possible amino acid residues of the activating protein which interact with apyrase, two relatively specific amino acid modifiers were employed. The participation of thiol groups can be excluded because of the insensitivity of Fr-A activating properties towards DTNB (Table 4). The treatment of Fr-A with TNM decreased its activating action on apyrase (Table 4). These results suggest that some tyrosyl residues of this regulatory protein may be essential for the interaction with apyrase. It can be considered that electrophilic attack of TNM is in this case specific for tyrosyl groups because the results with DTNB showed that participation of thiol groups was unlikely. The inhibitory protein could not be characterized due to the low yield and to its instability.

Two apyrase effector proteins which differ from calmodulin, obtained from the same source, are described for the first time in this paper. This is a very important finding that may open further approaches to the metabolic role of apyrase in the potato tuber. Although the hydrolytic activity of apyrase is its only well known enzyme function, it is possible that this may not be its unique role. The two proteins described, one of which is the only activating effector known so far, may be physiological modulators of this enzyme.

Table 4. Effect of modifying reagents on apyrase activator protein

Addition to the apyrase preincubation mixture.	ATPase (%)
Activator	53
DTNB treated BSA	0
DTNB treated activator	48
TNM treated BSA	0
TNM treated activator	30

ATPase activity of apyrase was assayed as described in the Experimental section. All controls were done at equivalent protein concentration of BSA to the activator added.

EXPERIMENTAL

Materials. All chemicals were reagent grade. Bovine heart PDE, bovine heart CaM and 5'-adenylic acid deaminase were obtained from Sigma.

Methods. Assay of calmodulin. Calmodulin was assayed by the stimulation of the activity of 'activator deficient' cyclic 3',5'-monophosphate phosphodiesterase (PDE) from bovine heart [12]. Assay conditions were the following: 16 mM cAMP, 30 mM Tris–HCl pH 8, 4 mM Ca^{2+}, 4 mM Mn^{2+} and 100 μg of phosphodiesterase. After 20 min at 30° the reaction was stopped by heating for 2 min at 100°. The generated AMP was deaminated to IMP. The reaction was followed by the decrease in A at 265 nm according to Sigma Instructions. This coupled assay was performed by addition of 0.38 U of 5'-adenylic acid deaminase in 10 mM Na citrate pH 6.5; the reaction was completed after 5 min of incubation at 25°.

Assay of apyrase activating and inhibitory fractions. These fractions were assayed by the extent of activation or inhibition of apyrase under standard assay conditions. The standard assay medium for apyrase activity contained 2 mM ATP (or ADP), 5 mM $CaCl_2$ and 100 mM sodium succinate pH 6 according to ref. [18]. The Pi produced after 5 min of incubation at 30° was measured by the method of ref. [19]. Apyrase was preincubated with the activating or with the inhibitory fraction or with BSA (as control) in the presence of either 0.1 M NaCl or 0.1 M buffer. Preincubation at different pH values was performed in the following buffer soln: NaOAc pH 5, MES pH 6, MES pH 7, and TES pH 8. The stimulating effect was completed in less than 10 s of preincubation at 0°. Aliquots of this mixture were assayed for apyrase activity as described above.

Protein determination. Protein content was measured by the method of ref. [20] and protein eluted from the column was estimated by A_{280}.

Purification of protein modulators. Peeled potatoes (*Solanum tuberosum* L. cv Ultimus 1 kg) were homogenized in a Waring blendor at maximum speed for three periods of 30 sec each in 2 l of 20 mM Tris–HCl, pH 7.5. This extract was filtered under vacuum through Whatman paper No. 4. The relatively clear filtrate was adjusted to 50% satn with solid $(NH_4)_2SO_4$. The ppt was removed by centrifugation at 17 300 g for 30 min. This material, dissolved in 200 ml of the extraction buffer, was heated at 100° for 5 min and rapidly cooled in ice. The insoluble material produced by the heating was separated by centrifugation at 17 300 g for 30 min. The supernatant was freeze dried and dissolved in 80 ml of H_2O and then exhaustively dialysed, against the equilibrium buffer described in the following section. This fraction (Fr-50) sometimes required centrifugation before using it for affinity chromatography.

An aliquot of 15 ml Fr-50 was applied at room temp. to a trifluoperazine-Sepharose column (1.5 × 27 cm) equilibrated with 10 mM Tris–HCl, 0.5 mM $CaCl_2$, pH 7. After applying the samples the column was washed with *ca* 230 ml of equilibrium buffer, followed by 0.5 M NaCl in the same buffer (300 ml) until the A at 280 nm was less than 0.005. Potato calmodulin was eluted with 10 mM Tris–HCl, 5 mM EGTA, pH 8. The fractions were assayed for their effect on phosphodiesterase and apyrase.

The three active peaks were concd by freeze-drying followed by dialysis against 0.1 M NaCl.

Binding of trifluoperazine to Sepharose 4B. Trifluoperazine was coupled to Sepharose 4B using the bisoxirane method described in ref. [21].

Gel electrofocusing. The purity of the protein samples was checked by isoelectric focusing according to ref. [22]. In the homogeneous protein the isoelectric point was determined by the same method.

SDS electrophoresis. The homogeneity and M_r of purified samples were estimated by SDS-PAGE [23].

Modification of amino acid residues of the apyrase activator. Reaction with DTNB (2.5 mM DTNB in 40 mM glycyl-glycine pH 8) was performed at 0° for 15 min according to ref. [24]. Chemical modification of apyrase activator (Fr-A) by TNM was done by the method described by ref. [25]. The reaction was carried out at room temp. for 1 hr in 60 mM Tris–HCl pH 8 in the presence of 10 mM TNM. The reaction was quenched by lowering the pH to 6. The stimulating effect of this modified activator was tested on apyrase.

It was necessary to take some precautions with the excess of TNM remaining in Fr-A after chemical modification because apyrase also has tyrosyl residues essential for ATPase and ADPase activity [9, 7]. Therefore, before mixing apyrase with TNM treated Fr-A, the pH was acidified from 8 to 6, under these conditions TNM reagent does not react with tyrosyl group [25].

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