PURIFICATION AND IDENTIFICATION OF CALMODULIN FROM *NEUROSPORA CRASSA*

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1. Introduction

The calcium-dependent regulatory protein calmodulin first described [1,2] as an activator of the cAMP-phosphodiesterase (PDE) in the bovine brain has been found in a variety of eucaryotic organisms. Calmodulin isolated from some fungi [3–6] and plants [7–9] seems to be similar to mammalian calmodulin [10].

Calmodulin has not yet been reported in Ascomycetes and calmodulin-like activity could not be detected in the yeast *Saccharomyces cerevisiae* [5].

Here, we describe the use of an affinity chromatography column with an immobilized chlorpromazine derivative [11] which enabled us to isolate *Neurospora crassa* calmodulin. It was identified on the basis of its electrophoretic properties, its activation of bovine heart PDE and its absorption spectrum.

2. Materials and methods

The wild-type strain STA_4 262A of *Neurospora crassa* was obtained from the Fungal Genetic Stock Center, Humboldt State University (Arcata CA). Erlenmeyer flasks (2.5 l) containing 1 l Vogel's minimal medium [12] supplemented with 2% sucrose were inoculated with 10^9 conidia. Cultures were agitated in a rotary shaker (300 rev./min) in the dark at 25°C for 48 h, corresponding to the stationary phase of growth.

Mycelia were harvested by filtration and washed with double-distilled water on a Büchner funnel. Mycelial pads were cut with scissors, then disrupted in a Moulinex chopper (type 320) for 3 min. Extraction buffer (40 mM Tris–HCl, 1 mM EDTA and 1 mM 2-mercaptoethanol, pH 7.0) was used at 1 ml/g mycelia wet wt. The homogenate was filtered through a single layer of nylon cloth, the remaining fragments of mycelia were recovered and retreated in half a volume of extraction buffer. This homogenate was pooled with the first filtrate. The pooled material was centrifuged at 48 000 × *g* for 30 min in the fixed-angle rotor SS-34 with a Sorvall RC-5B centrifuge. All steps were carried out at 4°C.

The resulting supernatant was mixed with DEAE–Sephacel and filtered on a sintered glass funnel by gravity. The filtrate was passed again on the resin and recovered by suction. The bulk of loaded proteins was released by subsequent washings with 100 mM NaCl in the extraction buffer. Further washings with 500 mM NaCl in the same buffer released another mixture of proteins (~15% of the loaded proteins) containing calmodulin. $CaCl_2$ was added to the eluate to a final concentration of 5 mM. The suspension was clarified by centrifugation at 48 000 × *g* for 30 min.

The supernatant was loaded on a chlorpromazine–Sepharose 4B affinity chromatography column prepared as in [11]. Extensive washings with 5 mM $CaCl_2$–500 mM NaCl in the extraction buffer was performed. Calmodulin was then released by 10 mM EGTA added to the buffer instead of 5 mM $CaCl_2$. Fractions of 1 ml were collected. All steps were monitored at 280 nm with a Fractoscan recorder system (Buchler).

Proteins were determined as in [13] using bovine serum albumin as standard. Calmodulin contents in the elution fractions of the affinity column were determined by electrophoresis of aliquots on 12.5% polyacrylamide slab gels using calmodulin from bovine brain as standard [14] and electrophoresis in the presence of sodium dodecyl sulfate (SDS) was

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performed as in [15]. The surface of each band was determined with a planimeter (Numonics model 1224 electronic digitizer) on the tracings of direct gel scanning in a Vitatron densitometer MPS type 940 000.

Bovine heart PDE activity was measured as in [16]. Verification of calcium-dependent activation of PDE in the presence of a saturating amount (1 μg) *Neurospora* calmodulin was performed by the addition of EGTA at 2 mM final conc.

CNBr-activated Sepharose 4B and DEAE–Sephacel were purchased from Pharmacia (Switzerland). Bovine heart PDE was obtained from Sigma (Switzerland). All other reagents were the best grades commercially available.

3. Results and discussion

Neurospora crassa calmodulin fractions obtained by affinity chromatography were analysed on SDS–12.5% polyacrylamide slab gel (fig.1) in the presence of either 5 mM $CaCl_2$ or 5 mM EGTA and compared to calmodulin from bovine brain. Calmodulin shows a characteristic calcium-dependent shift in its electrophoretic migration. The difference in migration produced by the addition of calcium compared to EGTA

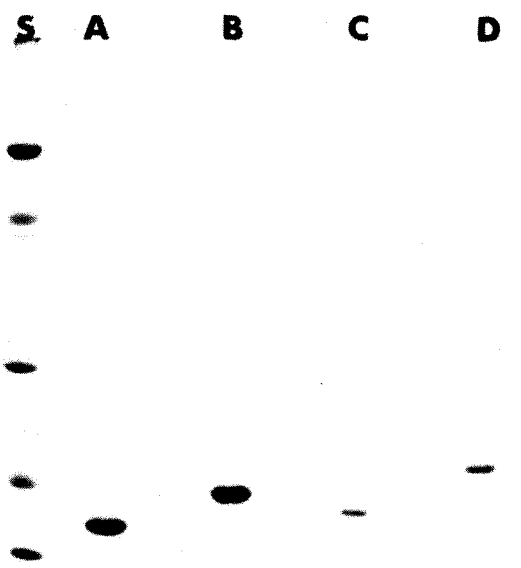

Fig.1. SDS–polyacrylamide gel electrophoresis of *Neurospora crassa* and bovine brain calmodulin. (A) *N. crassa* and (C) bovine brain calmodulin in the presence of 5 mM $CaCl_2$. (B) *N. crassa* and (D) bovine brain calmodulin in the presence of 5 mM EGTA. Protein standards (Sigma) (S): lysozyme (14 300 M_r), β-lactoglobulin (18 400 M_r), trypsinogen (24 000 M_r), pepsin (34 700 M_r), ovalbumin (43 000 M_r) and bovine serum albumin (67 000 M_r).

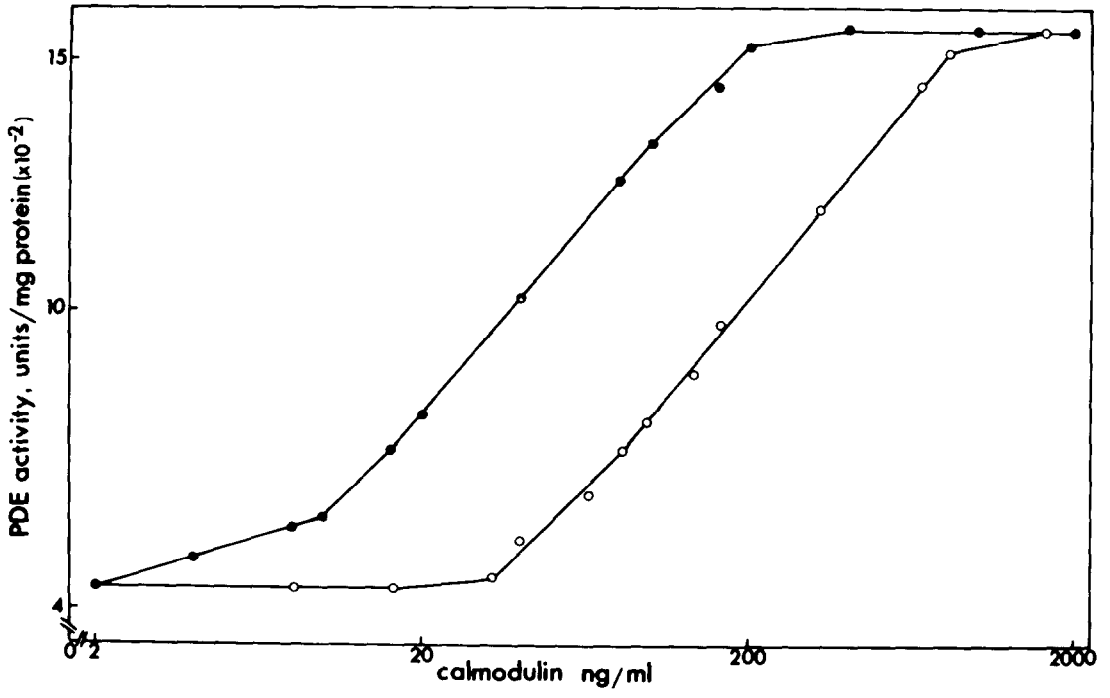

Fig.2. Activity of bovine heart PDE in the presence of various amounts of *Neurospora* (○—○) or bovine brain (●—●) calmodulin.

was the same for both *Neurospora* (fig.1, traces A,B) and bovine brain (traces C,D) calmodulin.

In the presence of calcium the app. M_r of *Neurospora* calmodulin as calculated from fig.1 was slightly lower than that of bovine brain, i.e., 16 000 compared to 17 000. Lower M_r-values than those of bovine brain calmodulin have been reported for fungal and plant calmodulin [10]. Fig.1 illustrates also the degree of purity of *N. crassa* calmodulin purified on a chlorpromazine–Sepharose 4B column. Major contaminations could not be detected.

Calmodulin recovery under our extraction conditions was ~0.75 mg/g total proteins; further analysis will be carried out to determine exactly the calmodulin content of mycelia from *Neurospora*.

Fig.2 shows the PDE activity as a function of increasing amounts of calmodulin from *Neurospora* and bovine brain. Maximal activation of PDE was reached in the presence of calcium and 800 ng *Neurospora* calmodulin. The PDE activity was not stimulated if only calcium or only calmodulin were present (not shown). Half-maximal activation of PDE was obtained with 180 ng/ml *Neurospora* and 36 ng/ml bovine brain calmodulin. From these data it is not possible to distinguish whether this difference is due to different functional properties of *N. crassa* calmodulin or to inactive molecules present in our preparation. Interestingly, the requirement of higher concentrations of calmodulin to obtain half-maximal stimulation of bovine brain PDE was also reported for *Dictyostelium discoideum* [5]. It was concluded [5] that a lower affinity of *Dictyostelium* calmodulin to bovine brain PDE was responsible for such results.

The absorption spectrum of *Neurospora* calmodulin is shown in fig.3. The peaks are located at 276, 268, 264, 258 and 252 nm, respectively and are in agreement with those reported for bovine heart calmodulin [17]. However, the relative extinction values of the peaks of *N. crassa* calmodulin are different from those of animal calmodulin and resemble very much those of plant calmodulin [8,18].

The above data clearly demonstrate the existence of calmodulin in *Neurospora crassa*. The electrophoretic mobility on polyacrylamide gels, the activation of bovine heart PDE and the absorption spectrum of *Neurospora* calmodulin are different from animal calmodulin and are similar to those of plant calmodulin.

We are now attempting to purify significant amounts of calmodulin from *N. crassa* to characterize its biochemical properties.

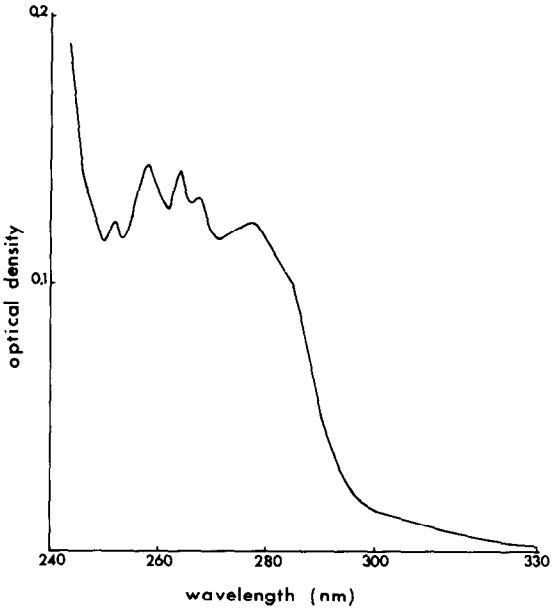

Fig.3. Absorption spectrum of *Neurospora crassa* calmodulin. The spectrum was obtained with calmodulin in a solution of 40 mM Tris–HCl, 500 mM NaCl, 10 mM EGTA (pH 7.0) to which $CaCl_2$ was added in excess in comparison to EGTA. The peaks are located at 276, 268, 264, 258 and 252 nm. The absorbance at 280 nm for 1 mg freeze-dried calmodulin/ml double-distilled water was 0.2.

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