LACK OF TISSUE SPECIFICITY OF CALMODULIN: A RAPID AND HIGH-YIELD PURIFICATION METHOD

Shiro KAKIUCHI, Kenji SOBUE, Reiko YAMAZAKI, Jun-ichi KAMBAYASHI+, Masato SAKON+ and Goro KŌSAKI+

Institute of Higher Nervous Activity, Osaka University Medical School, Kita-ku, Osaka 530 and *+The Second Department of Surgery, Osaka University Medical School, Fukushima-ku, Osaka 553, Japan*

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1. Introduction

The discovery of Ca^{2+}-activatable phosphodiesterase [1] led to the discovery of the modulator protein which confers the Ca^{2+}-sensitivity upon this enzyme [2,3]. An activator of phosphodiesterase was independently reported. The identity of both proteins was established subsequently [5]. This modulator protein nowadays termed calmodulin exhibits the Ca^{2+}-dependent activation of a number of enzymes and is now regarded as an intracellular mediator of actions of Ca^{2+} [6]. Its ubiquitous distribution in the animal and plant kingdoms and its structural and functional conservativeness throughout molecular evolution are well established [6,7].

Although there is general agreement concerning the gross similarities among vertebrate calmodulins, minor differences in structure and mobility upon polyacrylamide gel electrophoresis were seen between calmodulin preparations from rat testis [8], bovine uterus [9] and bovine brain [10,11]. It is thought that these differences are rather artifactual or due to mistaken assignments of the amino acid sequence, but this has not yet been proved or disproved. The present study supports the proposal that there is no difference between calmodulins from different tissues. It also describes a convenient purification method for calmodulin giving an overall recovery of >70%.

Abbreviations: PMSF, phenylmethyl-sulfonylfluoride; FOY, {ethyl-*p*'-(6'-guanidinohexanoyloxy)benzoate} methane sulfonate; EGTA, ethyleneglycol bis(β-aminoethylether)-*N*,*N*'-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid

2. Materials and methods

2.1. *Materials*

PMSF and DFP were from Sigma Chemicals (St Louis MO). Leupeptin, pepstatin A, antipain were from the Peptide Institute (Osaka). Epoxy-activated Sepharose 6B was from Pharmacia (Japan). Fluphenazine maleate and FOY were generous gifts of the Yoshitomi and Ono Pharmaceutical companies, respectively.

2.2. *Preparation of fluphenazine–Sepharose*

Epoxy-activated Sepharose 6B was soaked with deionized water and then washed with a coupling reaction medium consisting of 0.1 M carbonate buffer (pH 11) and dioxane at 20% (v/v) final conc. Washed epoxy-activated Sepharose (5 ml) was incubated for 48 h at 50°C in the above reaction medium containing 400 mg fluphenazine maleate in 20 ml and final pH 11: pH was readjusted by the addition of NaOH. The incubation was carried out in an incubator with shaking. At the end of the incubation, the Sepharose slurry was washed on a sintered glass funnel with the reaction medium and then with deionized water. It was then suspended in 2–3 resin volumes of 1 M ethanolamine (pH 8.0), followed by filtration on a sintered glass funnel. This procedure was repeated several times and the total incubation time with ethanolamine was ≥2 h. The ethanolamine-treated Sepharose was washed with deionized water and then with 20 mM Tris–HCl (pH 7.5) on a sintered glass funnel. Care was taken to minimize the exposure to light of the fluphenazine-coupled Sepharose during the coupling reaction and subsequent storage.

2.3. *Purification of platelet calmodulin*

Fresh bovine blood containing 1/10th vol. 5% dextrose solution–77 mM EDTA to prevent coagulation was centrifuged at 250 × *g* for 10 min. The resultant supernatant (platelet rich plasma) was then centrifuged at 1500 × *g* for 5 min. The pellet was resuspended in Ca^{2+}- and glucose-free Tyrode's solution containing 1 mM EGTA. The suspension was recentrifuged as above. This washing procedure was repeated twice and final pellet was resuspended in 20 mM Tris–HCl (pH 7.4), 1 mM EGTA, 150 mM NaCl, 0.5 mM FOY, 1 mM PMSF, pepstatin A (0.1 μg/ml), antipain (5 μg/ml). This mixture was kept frozen at –80°C until use. The frozen pellet of platelets (30 ml) was homogenized with 3 vol. (90 ml) 4.0% (w/v) trichloroacetic acid. After 20 min gentle stirring, the mixture was centrifuged at 20 000 × *g* for 30 min. The resultant pellet was homogenized with 125 ml medium A consisting of 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.1 mM diisopropylfluorophosphate, pepstatin A (0.05 μg/ml) and antipain (2 μg/ml). The pH of this homogenate was adjusted to 7.5 by the dropwise addition of 6 M NaOH and the homogenate was centrifuged at 105 000 × *g* for 60 min. The pellet was rehomogenized with 30 ml medium A and recentrifuged as above. The supernatant fluids derived from these 2 homogenates (120 ml, 27 ml) were combined and applied to a column (1.5 × 11.5 cm) of DEAE-cellulose which had been equilibrated with 20 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 0.1 mM EGTA (medium B). The column was eluted with medium B plus 0.145 M $(NH_4)_2SO_4$ (at I →) and then with medium B plus 0.3 M $(NH_4)_2SO_4$ (at II →) (fig.3a). Fractions of 4 ml each were collected. Fractions containing calmodulin activity were combined (tubes no. 106–112) and diluted with an equal volume of 20 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol and 0.5 mM $CaCl_2$ to make 0.15 M in $(NH_4)_2SO_4$ and 0.2 mM in free Ca^{2+}. The diluted solution was applied to a column (0.8 × 1.2 cm) of the fluphenazine–Sepharose 6B. The column, which had been equilibrated with 20 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 500 mM NaCl and 0.2 mM $CaCl_2$, was eluted with the above wash medium. At arrow III, the elution medium was switched to 20 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 500 mM NaCl and 2 mM EGTA (fig.3b). Fractions of 4 ml each (tubes no. 1–35) or 1 ml each (tubes no. 36–) were collected. Fractions containing calmodulin were combined (tubes no. 36–40) and saved. The above procedures were done at 4°C.

2.4. *Purification of calmodulin from other sources*

Fresh bovine brain and testis were obtained from a local slaughterhouse. A thermotolerant strain of *Tetrahymena pyriformis* (NH-I) was kindly provided by Dr Y. Nozawa, Gifu University. The cells collected by centrifugation were frozen in dry ice and transported to the laboratory in dry ice. These materials were homogenized in Waring Blendor with 3 vol. 4.0% trichloroacetic acid. With the frozen samples trichloroacetic acid was added while the samples were still frozen, this addition being immediately followed by the homogenization. Calmodulin was purified from these homogenates by essentially those procedures described for platelet, except that medium A was altered to 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.1 mM diisopropylfluorophosphate, pepstatin A (0.05 μg/ml), and PMSF (50 μg/ml). For *Tetrahymena* samples, final concentrations of diisopropylfluorophosphate and pepstatin A were 1 mM and 0.5 μg/ml, respectively.

2.5. *Analytical procedures*

Calmodulin was determined according to its ability to activate the calmodulin-deficient brain phosphodiesterase [12]. Values were corrected as in [13]. Protein was determined as in [13]. Electrophoresis of proteins was done in 15% polyacrylamide gels in the buffer system of Davis [14] (50 mM Tris–384 mM glycine, pH 8.3), or in 0.1% SDS in a Laemmli buffer system [15]. When system of Davis was employed, either EGTA or $CaCl_2$ was added to both the gels and the samples at 0.2 mM final conc.

3. Results and discussion

These studies were undertaken to determine whether calmodulin preparations from different tissues of the same species (bovine) were identical. Calmodulins were purified to homogeneity from bovine platelet, testis and brain as judged by polyacrylamide gel electrophoresis in three different conditions, i.e., in SDS gel and in non-denaturing gels in the presence and absence (+EGTA) of Ca^{2+} (fig.1a,b). Calmodulin showed a characteristic mobility change on non-denaturing gels depending upon the presence and absence of Ca^{2+} in the gels. The decreased mobility of

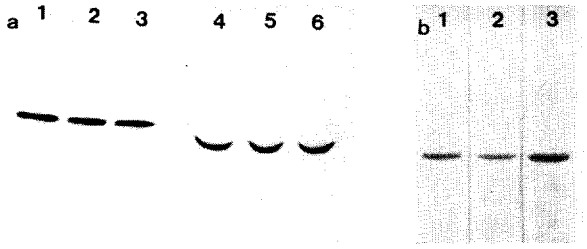

Fig.1. Comparison of calmodulins upon polyacrylamide gel electrophoresis. (a) Results with platelet and brain proteins using the buffer system of Davis [14] containing either 0.2 mM $CaCl_2$ (1–3) or EGTA (4–6): (1,4) 2 μg of platelet protein; (2,5) 2 μg brain protein; (3,6) 1 μg each of both proteins. Identical results were obtained with testis and brain proteins (not shown). (b) Results with testis and brain proteins using a buffer system of Laemmli [15] containing 0.1% SDS: (1) 2.5 μg brain protein; (2) 2.5 μg testis protein; (3) 2.5 μg each of both proteins. Identical results were obtained with platelet and brain proteins (not shown).

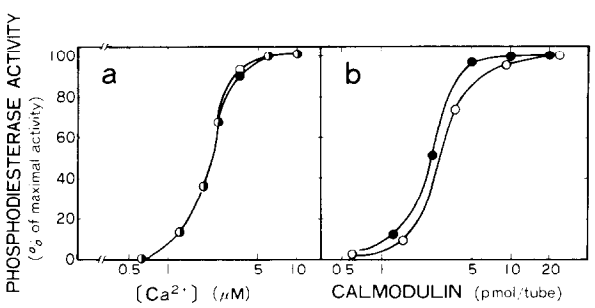

Fig.2. Ca^{2+}-dependent activation of the calmodulin-deficient brain phosphodiesterase activity by platelet (○) and brain (●) calmodulins. In (a), calmodulin was 0.35 μg/assay tube (0.5 ml). Ca^{2+}-EGTA mixture was used to produce the indicated concentrations of free Ca^{2+} [12]. In (b), all assay tubes contained 0.1 mM $CaCl_2$.

calmodulin in the presence of Ca^{2+} contrasts with the increased mobility of troponin C [16] and leiotonin C [17] and also with the mobility of the majority of other cellular proteins which are unaffected by Ca^{2+}. Therefore, polyacrylamide gel electrophoresis can be used as a reliable identification method for calmodulin [12]. It also acts as a sensitive purity check of calmodulin. Calmodulin preparations from platelet and testis coincided with brain calmodulin in each of the 3 different electrophoretic systems (fig.1a,b). In [8] rat testis calmodulin and bovine brain calmodulin separated into two distinct bands on SDS gel electrophoresis. The reason for this discrepancy is not clear at present.

Another criterion for the identification of calmodulin is its ability to activate phosphodiesterase. Calmodulin preparations of platelet, testis and brain were indistinguishable in their Ca^{2+}-dependent activation of the calmodulin-deficient brain phosphodiesterase activity (fig.2). Thus, the results are consistent with the proposal that there is no tissue difference of calmodulin.

With the purification method used here, yields of calmodulins from bovine platelet, brain and *Tetrahymena* were 49, 200 and 70 μg, respectively, per ml cells (platelet and *Tetrahymena*) or per g tissue (brain). Since we determined the calmodulin content of these cells to be ~60, 300 and 100 μg, respectively, per ml or g, above yield values represent the recovery rate of ~70% for all cases, the highest rate ever reported. Although there are a variety of published methods for the purification of calmodulin, most are time-consuming and give low recoveries. The phenothiazine affinity chromatography developed in [18,19] is certainly superior to the other methods in its simplicity. However, there was only a slight improvement in its overall recovery as compared to other methods. The purification method used here is therefore described in detail in sections 3.1–3.3; the 3 step procedure can be accomplished within 3 days. Application of this method to bovine platelet is summarized in table 1.

3.1. *Trichloroacetic acid treatment*

It was essential to guard against the proteolytic attack on calmodulin during the initial homogenization and subsequent procedures, because some of the proteolytic products of calmodulin behaved like calmodulin upon column chromatography and were poorly separated from the latter. Attempts to destroy the proteolytic activity by heat-treatment of homogenates often resulted in the increased production of the proteolytic fragments. During the initial rise in temperature an activated proteolysis may have taken place. Making the homogenate with trichloroacetic acid and inclusion of protease inhibitors in the subsequent step gave a satisfactory result. The trichloroacetic acid treatment from [20] was modified so that the recovery at this step was ~80%.

3.2. *DEAE-cellulose column chromatography*

After the sample application, the column was washed by a medium containing 0.145 M $(NH_4)_2SO_4$.

Table 1
Summary of purification of calmodulin from bovine platelets

	Protein (mg)	Calmodulin		Yield (%)
		(μg/mg protein)	(μg)	
Homogenate	1089[a]	1.8	1960[a]	100
Trichloroacetic acid supernatant	35	n.d.[b]	n.d.	–
Trichloroacetic acid precipitate	1005	1.5	1507	77
Extract from the trichloroacetic acid precipitate	145	10	1450	74
Residue after the above extraction	851	n.d.	n.d.	
DEAE-cellulose column	18	81	1458	74
Fluphenazine–Sepharose column	1.4	1040	1456	74

[a] Derived from 30 ml packed volume; therefore, calmodulin in the original homogenate was 63 μg/ml packed volume
[b] n.d., not detectable

Many proteins were eluted from the column by this wash. Calmodulin was then eluted from the column by raising the concentration of $(NH_4)_2SO_4$ to 0.3 M (fig.3a). This 2-step elution instead of a gradient elution made the chromatography operation quite simple.

3.3. *Fluphenazine affinity chromatography*

The recovery of this step increased to ~100% (with brain sample) as compared to 55% with the original method [18]. We found that 2 factors are important in the high recovery:

(i) The calmodulin-binding proteins [21,22] interfere with the Ca^{2+}-dependent binding of the calmodulin to the resin. Here, the sample after the DEAE-cellulose column was devoid of such activity. The binding proteins must have been removed by the preceding procedures.

(ii) More importantly, the Sepharose matrix, though treated with ethanolamine, bound calmodulin non-specifically and irreversibly. Thus, the recovery decreased as the amount of calmodulin applied to the resin column was decreased (table 2). The degree of such non-specific binding of calmodulin was greater with the Sepharose which was not treated with phenothiazine than

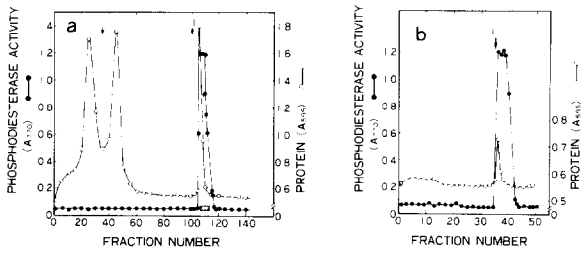

Fig.3. Purification of platelet calmodulin by column chromatographies. DEAE-cellulose column chromatography (a), and fluphenazine affinity chromatography (b) were done as in the text.

Table 2
Recovery of calmodulin upon fluphenazine affinity chromatography

Expt no.	Calmodulin applied		Calmodulin recovered	
	(μg)	(% of the total column capacity)	(μg)	(% recovery)
1	31	2.7	25	80
2	360	32	330	92
3	690	60	680	99

Various amounts of brain sample after DEAE-cellulose column, containing 91 μg/ml calmodulin, was applied to a 0.25 ml fluphenazine–Sepharose column (6 mm diam.). In a separate experiment, the total capacity of the column was calibrated to be 1.15 mg calmodulin. Calmodulin was determined according to its ability to activate brain phosphodiesterase (see section 2)

with the Sepharose treated with phenothiazine, since we observed that the recovery of calmodulin from a column containing a mixture of 1 part of fluphenazine–Sepharose and 3 parts of ethanolamine-treated Sepharose was 60% when the column was loaded with an amount of calmodulin corresponding to half the total column capacity.

On the basis of these observations, we modified the procedure of the affinity chromatography as follows:

(a) The amount of the calmodulin sample applied to the column was adjusted to at least 1/3rd of the capacity of the column;

(b) A large excess of fluphenazine was used for the coupling reaction when fluphenazine–Sepharose was synthesized.

The latter resulted in the increased capacity of the gel for calmodulin-binding compared to the original method [18] (4.6 mg *vs* 3 mg calmodulin/ml gel) and contributed to the decrease of the non-specific calmodulin-binding of the resin matrix. A typical elution profile of the affinity chromatography is shown in fig.3b. *Tetrahymena* calmodulin is slightly different from the mammalian protein [13], but it was eluted from the columns in exactly the same manner as the mammalian calmodulin.

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References

[1] Kakiuchi, S. and Yamazaki, R. (1970) Proc. Japan Acad. 46, 387–392.
[2] Kakiuchi, S., Yamazaki, R. and Nakajima, H. (1970) Proc. Japan Acad. 46, 587–592.
[3] Kakiuchi, S. and Yamazaki, R. (1970) Biochem. Biophys. Res. Commun. 41, 1104–1110.
[4] Cheung, W. Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.
[5] Teo, T. S. and Wang, J. H. (1973) J. Biol. Chem. 245, 5950–5955.
[6] Means, A. R. and Dedman, J. R. (1980) Nature 285. 73–77.
[7] Klee, C. B., Crouch, T. H. and Richman, P. G. (1980) Annu. Rev. Biochem. 49, 489–515.
[8] Dedman, J. R., Patter, J. D., Jackson, R. L., Johnson, D. and Means, A. R. (1977) J. Biol. Chem. 252, 8415–8422.
[9] Grand, R. J. A. and Perry, S. V. (1978) FEBS Lett. 92, 137–142.
[10] Watterson, D. M., Sharief, F. and Vanaman, T. C. (1980) J. Biol. Chem. 255, 962–975.
[11] Kasai, H., Kato, Y., Isobe, T., Kawasaki, H. and Okuyama, T. (1980) Biomed. Res. 1, 248–264.
[12] Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M. and Uenishi, K. (1978) J. Biol. Chem. 253, 1338–1340.
[13] Kakiuchi, S., Sobue, K., Yamazaki, R., Nagao, S., Umeki, S., Nozawa, Y., Yazawa, M. and Yagi, K. (1981) J. Biol. Chem. 256, 19–22.
[14] Davis, B. J. (1964) Ann. NY Acad. Sci. 121, 404–427.
[15] Laemmli, U. K. (1970) Nature 227, 680–685.
[16] Grand, R. J. A., Perry, S. V. and Weeks, R. A. (1979) Biochem. J. 177, 521–529.
[17] Mikawa, T., Nonomura, Y., Hirata, M., Ebashi, S. and Kakiuchi, S. (1978) J. Biochem. 84, 1633–1636.
[18] Charbonneau, H. and Cormier, M. J. (1979) Biochem. Biophys. Res. Commun. 90, 1039–1047.
[19] Jamieson, G. A. and Vanaman, T. C. (1979) Biochem. Biophys. Res. Commun. 90, 1048–1056.
[20] Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. 87, 1313–1320.
[21] Wang, J. H. and Desai, R. (1977) J. Biol. Chem. 252, 4175–4184.
[22] Sharma, R. K., Wirch, E. and Wang, J. H. (1978) J. Biol. Chem. 253, 3575–3580.