Journal of Chromatography, 363 (1986) 456–461
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 831

Note

High-pressure affinity chromatography of calmodulin on a phenothiazine-silica

HARRY W. JARRETT
Department of Biology, Purdue University School of Science, 1125 E. 38th Street, Indianapolis, IN 46223 (U.S.A.)
(Received May 29th, 1986)

Calmodulin is an acidic, Ca^{2+}-dependent protein activator of a variety of enzymes[1]. Also in the presence of Ca^{2+}, calmodulin binds phenothiazine antidepressants[2] and other lipophilic compounds[3]. Drug binding antagonizes enzyme activation by calmodulin. This Ca^{2+}-dependent binding of antagonists has also provided the basis for affinity chromatography of calmodulin.

Low-pressure affinity chromatography (LPAC) of calmodulin on phenothiazine-Sepharoses[4–7], W-7-Sepharose[8] and phenyl-Sepharose[9] has been used to purify calmodulin and calmodulin-like proteins from a variety of plant, animal, fungal and protozoan sources. High-pressure affinity chromatography (HPAC) on silica-based resins is a fairly recent development[10,11], which has the promise of decreased analysis time, higher resolution and thus greater sensitivity over LPAC. Here, we describe 2-(trifluoromethyl)-10-(3′-aminopropyl)phenothiazine (TAPP)-silica, an HPAC resin which allows rapid and sensitive detection of calmodulin in crude tissue extracts.

EXPERIMENTAL

Synchropak CM-300 (6.5 μm bead, 300 Å pore) and 3-glycidyloxypropyl-silica (5 μm, 300 Å pore) were the kinds gifts of Alltech. (Deerfield, IL, U.S.A.). All reagents used were of analytical grade or of the highest purity obtainable. Calmodulin and phosphodiesterase were purified from bovine brain and assayed as previously described[12]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out on 12.5% acrylamide, 0.312% bisacrylamide gels by the method of Laemmli[13] except that all buffers contained 1 m*M* calcium chloride. Acid hydrolysis and amino acid analysis were performed as previously described[14]. TAPP was synthesized by the method of Hart *et al.*[15]. The chromatograph was a Varian 5000 instrument.

Synthesis of TAPP-silica

All operations were performed at room temperature (18°C). To 1.5 g of CM-300 resin were added 4.5 mmol dicyclohexylcarbodiimide, 9 mmol N-hydroxysuccinimide and 2.5 ml dioxane. The resin was deaerated by sonication under vacuum and shaken for 24 h. The resin was then washed thoroughly on a filter with hexane, dioxane, ethanol and, finally, 0.1 *M* sodium phosphate, pH 7, and suspended in 100

0021-9673/86/$03.50

ml of the same buffer containing 25 mmol β-Ala and allowed to react for 2 h. The resulting resin was washed thoroughly with 10 m*M* hydrochloric acid, water, acetone and, finally, dioxane. This resin was then reacted with dicyclohexylcarbodiimide and N-hydroxysuccinimide and thoroughly washed as described above. The resin was then suspended in 10 ml 0.1 *M* sodium phosphate, pH 7–dioxane (1:1) containing 0.28 mmol TAPP. The mixture was allowed to react overnight with shaking and the resin was then washed with dioxane and suspended in 10 ml 1 *M* ethanolamine, 0.1 *M* sodium phosphater, pH 7, to react with any reactive esters remaining on the resin. After reacting overnight, the resin was thoroughly washed with buffer, methanol and, finally, acetone and dried to constant weight at 50°C, yielding 1.2 g. A portion of the resin was acid hydrolysed[14], dried *in vacuo* and extracted with 1 ml ethanol; the concentration of phenothiazine was determined spectrophotometrially[12] to be 6.4 μmol TAPP/g resin. A portion of the ethanol extract was also subjected to amino acid analysis; the β-Ala concentration was found to be 34 μmol/g resin. The resin was packed into a 30 × 4.6 mm I.D. column under 475 bar from a dioxane slurry.

RESULTS AND DISCUSSION

The resin synthesized is depicted in Fig. 1. The carboxymethyl-silica starting material was first derivatized with β-Ala to provide an additional four-atom chain spacer to which was then attached TAPP. The columns packed with this resin were then tested.

In the experiment presented in Fig. 2, a crude extract of bovine brain was loaded onto the column and the column was washed and eluted. Fractions were collected and assayed for calmodulin activity with the phosphodiesterase assay. The results clearly show that calmodulin present in the sample was retained on the column and was not eluted by Ca^{2+}-containing buffers (A and B, Fig. 2) but was eluted when ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) replaced Ca^{2+} (C, Fig. 2). Calmodulin activity was detected only in a peak with a retention time close to that of the peak of protein eluted by the EGTA-containing buffer.

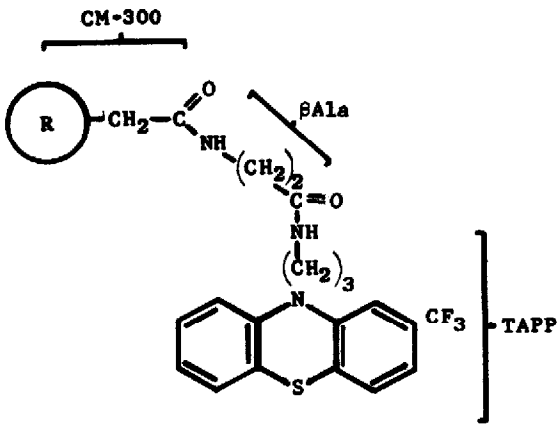

Fig. 1. TAPP-silica.

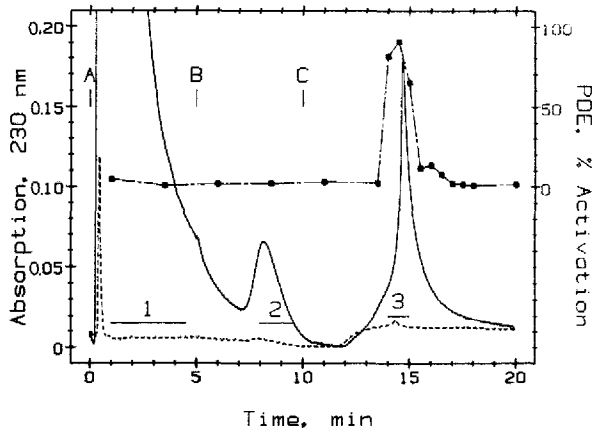

Fig. 2. Isolation of calmodulin from bovine brain extracts required less than 16 min. Frozen bovine brain was homogenized in 4 ml/g of 10 m*M* Tris–HCl, 2 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), pH 7.5 with a Tekmar homogenizer. The homogenate was filtered through cheesecloth and centrifuged at 100 000 *g* for 30 min. The supernatant was decanted, mixed with 100 m*M* calcium chloride to obtain a final concentration of 2.5 m*M* calcium, and 500 μl were injected onto the 30 × 4.6 mm I.D. TAPP-silica column. Prior to chromatography, the column was equilibrated in buffer A (250 m*M* sodium sulfate, 10 m*M* sodium phosphate, 1 m*M* calcium chloride, 0.5 m*M* EGTA, pH 6.5) which was continued for the first 5 min after injection, then the elution buffer was rapidly changed by 5.1 min to buffer B (same as buffer A but no sodium sulfate). Elution with buffer B was continued until 10 min when the buffer was rapidly changed by 10.1 min to buffer C (10 m*M* sodium phosphate, 1 m*M* EGTA, pH 6.5) which was maintained until the end of the run (buffer changes indicated by A, B and C on figure). The flow-rate was 2 ml/min throughout. 0.5-min Fractions were collected and 5-μl portions (or 5 μl of buffer A) were added to the 0.5-ml phosphodiesterase (PDE) assay. The enzyme results (●— · —●) are presented as the percent increase in enzyme activity normalized to the activity measured with 5 μg calmodulin defined as 100% activation. A baseline (----) recorded immediately following the run is also included for comparison.

Other experiments (data not shown) with calmodulin standards under the chromatographic conditions given in Fig. 2 demonstrated that the area of the EGTA-eluted peak was linearly related to the amount of calmodulin loaded up to 75 μg calmodulin (the highest amount tested); linear least squares analysis of the data gave a correlation coefficient of 0.998, and the limit of detection (signal-to-noise ratio) at 230 nm was 0.2 μg calmodulin (assuming noise of 0.001 absorbance units). Based on peak area, the brain extract (500 μl, Fig. 2) contained 33.6 μg calmodulin.

Fractions were pooled as shown in Fig. 2, and analysed by electrophoresis (Fig. 3). Fig. 3 clearly shows that the EGTA-eluted fractions (pool 3) contain only a single protein species that migrates upon electrophoresis like authentic calmodulin. The apparent homogeneity is especially significant since brain extracts such as this would contain S-100 (M_r 10 500), a protein that binds to some LPAC columns[8] and contaminates the calmodulin obtained; such was not the case with TAPP-silica. An aliquot of pool 3 was also acid hydrolyzed and the amino acid composition was determined[14]. The composition (data not shown) was virtually identical to that of pure calmodulin, confirming the purity of the protein obtained, and allowed us to calculate that 25.9 μg of calmodulin was present in the EGTA pool. This value agrees reasonably well with the 33.6 μg determined on the basis of peak area (see above).

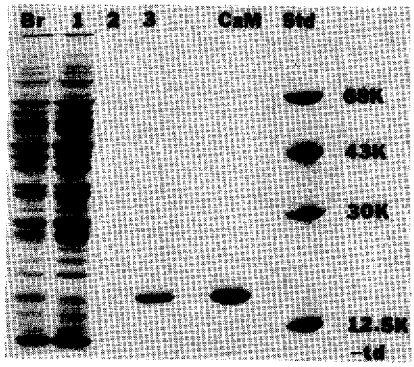

Fig. 3. The EGTA-eluted calmodulin appeared homogeneous upon electrophoresis. Fractions were pooled as indicated in Fig. 2 (indicated by bars numbered 1, 2, or 3), were dialysed exhaustively against 10 m*M* ammonium hydrogen carbonate, and lyophilized. The lyophilized protein was redissolved in 100 μl of water and 20 μl were saved for amino acid composition analysis (see text) and 40 μl was prepared in Laemmli sample buffer[13] and electrophoresed. The lane for pools 1, 2, and 3 are so indicated. 50 μl of a 1:10 dilution of the crude extract was run in lane Br, while 10 μg of pure bovine brain calmodulin was run in lane CaM for comparison. Molecular weight standards were run in the lane marked Std and are, in descending order, bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome *c*. The position of the bromophenol blue tracking dye is indicated by "td".

Thus, the separation of 500 μl of brain extract, corresponding to 125 mg of bovine brain, yielded calmodulin that appeared homogeneous by both physical (electrophoresis in sodium dodecylsulfate) and chemical (amino acid composition) criteria and the chromatographic separation required less than 16 min. This amount of calmodulin corresponds to about 200 mg calmodulin/kg brain which agrees well with the amount present in brain estimated by a variety of other methods[6,9,16,17].

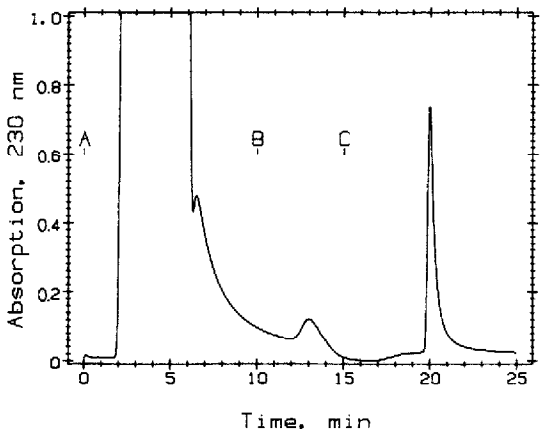

Fig. 4. The capacity was 0.58 mg calmodulin/ml resin under the conditions used. The column was equilibrated in buffer A prior to the run. At time zero 500 μl of 16.7 mg/ml calmodulin in buffer A was loaded. The flow-rate was 0.2 ml/min of buffer A for the first 5 min, after which the flow-rate was 2 ml/min and the elution program was identical to that given for Fig. 2. The times of buffer changes are indicated by A, B, and C.

The capacity of the TAPP-silica column was next investigated. When excess calmodulin was loaded at 0.2 ml/min and the column thoroughly washed and eluted, 0.29 mg calmodulin was recovered in the EGTA eluate. Loading different amounts of calmodulin or the use of different flow-rates, up to 2 ml/min, did not significantly alter the result. This corresponds to a capacity of 0.58 mg calmodulin/ml resin, which is about ten-fold lower than the capacity of TAPP-Sepharose[7].

The TAPP-silica column described allows the rapid separation and quantitation of calmodulin from crude tissue extracts. While the limit of detection (0.2 μg) is certainly much higher than that of the radioimmunoassay[16] or various enzyme assays[17], the calmodulin can be recovered from the HPAC column and further analysed by chemical and physical methods to confirm its identity; an approach not possible with other assays and often necessary when investigating calmodulin-like proteins. The speed of separation and sensitivity allow for rapid screening of small amounts of tissue.

The resin described here was not the only phenothiazine-silica prepared by us (unpublished data) or others[18]. We have also coupled TAPP to 3-glycidyloxypropyl-silica and have coupled 10-(3-propionyloxysuccinimide)-2-(trifluoromethyl)phenothiazine[12] to aminopropyl-silica. In both cases, more than 50 μmol phenothiazine/g resin was incorporated, but, in each case, elution of bound calmodulin required the use of organic solvents (*i.e.*, greater than 60% methanol) and no Ca^{2+}-dependence of retention time was observed. This behavior is indicative of a primarily reversed-phase interation between calmodulin and the stationary phase rather than an affinity interaction. This agrees well with the report of Marshak *et al.*[18] in which a phenothiazine-silica containing 1 mmol phenothazine per gram also behaved essentially as a reversed-phase resin. What makes the resin described here behave as an affinity resin rather than a reversed-phase one is unknown, but it appears clear that larger amounts of coupled phenothiazine and different substrate silicas can have unexpected effects.

ACKNOWLEDGEMENTS

The excellent technical assistance of William Foster is gratefully acknowledged. We also thank James Anderson and Alltech Associates, Inc., for the gift of resins and for packing many columns for us.

REFERENCES

1 W. Y. Cheung, *Science (Washington, D.C.)*, 207 (1980) 19–27.
2 W. C. Prozialeck and B. Weiss, *J. Pharmacol. Exp. Ther.*, 222 (1982) 509–516.
3 T. Tanaka, T. Ohmura and H. Hidaka, *Mol. Pharmacol.*, 22 (1982) 403–407.
4 H. Charbonneau and M. J. Cormier, *Biochem. Biophys. Res. Commun.*, 90 (1979) 1039–1047.
5 G. A. Jamieson and T. C. Vanaman, *Biochem. Biophys. Res. Commun.*, 90 (1979) 1048–1056.
6 C. R. Caldwell and A. Haug, *Anal. Biochem.*, 116 (1981) 325–330.
7 H. Charbonneau, R. Hice, R. C. Hart and M. J. Cormier, *Methods Enzymol.*, 102 (1983) 17–39.
8 T. Endo, T. Tanaka, T. Isobe, H. Kasai, T. Okuyama and H. Hidaka, *J. Biol. Chem.*, 256 (1981) 12485–12489.
9 R. Gopalakrishna and W. B. Anderson, *Biochem. Biophys. Res. Commun.*, 104 (1982) 830–836.
10 R. R. Walters, *Anal. Chem.*, 57 (1985) 1099–1114A.
11 J. N. Kinkel, B. Ansprach, K. K. Unger, R. Wieser and G. Brunner, *J. Chromatogr.*, 297 (1984) 167–177.

12 H. W. Jarrett, *J. Biol. Chem.*, 259 (1984) 10136–10144.
13 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
14 H. W. Jarrett, K. D. Cooksy, B. Ellis and J. M. Anderson, *Anal. Biochem.*, 153 (1986) 189–198.
15 R. C. Hart, M. D. Bates, M. J. Cormier, G. M. Rosen and P. M. Conn, *Methods Enzymol.*, 102 (1983) 195–204.
16 J. G. Chafouleas, J. R. Dedman, R. P. Munjaal and A. R. Means, *J. Biol. Chem.*, 254 (1979) 10262–10267.
17 A. C. Harmon, H. W. Jarrett and M. J. Cormier, *Anal. Biochem.*, 141 (1984) 168–178.
18 D. M. Marshak, T. J. Lukas, C. M. Cohen and D. M. Watterson, in D. Hartshorne and H. Hidaka (Editors), *Calmodulin Antagonists and Cellular Physiology*, Academic Press, New York, 1984, pp. 495–510.