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RAPID PURIFICATION OF CALMODULIN AND S-100 PROTEIN BY AFFINITY CHROMATOGRAPHY WITH MELITTIN IMMOBILIZED TO SEPHAROSE

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SUMMARY: Melittin-Sepharose was prepared for Ca^{2+}-dependent affinity chromatography of calmodulin and S-100 protein. This matrix exhibits extremely high capacity (~ 10 mg calmodulin/ml gel), low nonspecific binding, and excellent recovery (> 90%) under optimal conditions. Recovery of calmodulin from melittin-Sepharose was related to the degree of saturation of column capacity with lower yields when only partial saturation was achieved. Large-scale, simultaneous purification of calmodulin and S-100 protein from brain was carried out using selective adsorption to organomercurial agarose followed by melittin-Sepharose chromatography; yields were 250-300 mg of calmodulin and 200-300 mg of S-100 per kg tissue. Calmodulin also was purified in a single step from bovine testis supernatant using melittin-Sepharose in yields comparable to those from brain.

Melittin, a bee venom peptide, inhibits calmodulin (CaM)[1] activation of at least two enzymes, cyclic nucleotide phosphodiesterase (1,2) and myosin light chain kinase (3). In addition, the direct interaction of this peptide with CaM has been demonstrated by noting changes in its fluorescence anisotropy (4,5) and by quantification of the amount of complex formed using electrophoresis and gel filtration chromatography (1). Although there are some differences in the values reported, the affinity for CaM appears to be in the nanomolar range. Thus, the use of melittin for affinity chromatography of CaM was suggested. We describe here the preparation and properties of melittin-Sepharose and its application for the purification of CaM from crude extracts of brain and testis. In addition, a large-scale method for simultaneous purification of calmodulin and S-100 protein using organomercurial agarose and melittin-Sepharose chromatography is reported.

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[1] Abbreviations used in this paper: CaM, calmodulin; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N',N'tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; QAE, quaternary aminoethyl.

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Materials and Methods

Preparation of melittin-Sepharose. CNBr-activated Sepharose 4B (Pharmacia) was washed with 1 mM HCl as recommended by the manufacturer and suspended in an equal volume of 0.1 M sodium bicarbonate, pH 8.5, containing 0.5 M NaCl. An equal volume of melittin (Sigma), 1-20 mg/ml in the same buffer, was added and, after gently mixing for 1 h at room temperature, the gel was washed on a sintered glass funnel with the same buffer. An equal volume of 1 M ethanolamine·HCl, pH 8.0, was added and the mixture further incubated for 1 h at room temperature. The resulting melittin-Sepharose 4B was washed three times with 0.1 M sodium acetate, pH 4.5, containing 0.5 M NaCl followed by 0.1 M sodium borate, pH 9.0, containing 0.5 M NaCl, then washed with and stored in 25 mM BES, pH 7.0, containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM NaN_3, and 250 mM NaCl (buffer A) at 4°C. Melittin-Sepharose was routinely regenerated by washing with 0.1 M sodium acetate, pH 4.5, containing 6 M urea, 0.5 M NaCl and 10 mM EGTA as described for CaM-Sepharose (6).

Preparation of partially purified CaM. Brain CaM for chromatography experiments was partially purified using the first step of a procedure described for the purification of phosphodiesterase (7). After elution of the peak I phosphodiesterase from DEAE-BioGel A (Bio-Rad), the gel was washed with three bed volumes of the pH 5.45 buffer containing 100 mM NaCl and eluted with three bed volumes of the same buffer containing 400 mM NaCl. The eluate, referred to as partially purified CaM, was concentrated to ~ 1 mg protein/ml and used as the starting material for the chromatography on melittin-Sepharose. Ca. 50% of the protein in this fraction was CaM; the presence of a large amount of nucleotide was evidenced by very high absorbance at 260 nm.

For large-scale purification of CaM and S-100 protein, brain tissue was homogenized in four volumes of 25 mM BES containing 125 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EGTA and 6 M urea (freshly deionized) as described (7). Following brief centrifugation (10 min, 3000 x g), QAE-Sephadex A-25 (Pharmacia), 0.4 L/kg tissue, was added to the supernatant and stirred (2-3 h) after which the gel was allowed to settle. The gel was collected on a porous support (e.g., sintered glass funnel), washed with 1.2 bed volumes of homogenization buffer, followed by two bed volumes of that buffer lacking 6 M urea and eluted with three to four bed volumes of buffer containing 0.5 M NaCl. This QAE eluate was concentrated to ~ 1-4 mg protein/ml and applied to a bed of organomercurial agarose (Affi-Gel 501, Bio-Rad), ~ 80 ml/kg tissue, and washed with 1.2 bed volumes of buffer containing 0.25 M NaCl. The material not retained on this gel contained CaM while that eluted with 10 mM dithiothreitol contained S-100 protein; both fractions were made 2 mM with $CaCl_2$ and subsequently chromatographed on melittin-Sepharose to give homogenous proteins.

SDS gel electrophoresis. SDS gel electrophoresis was carried out on samples precipitated with 10% (w/v) trichloroacetic acid as described (8) except that the dimensions of the stacking and resolving gel were smaller (0.5 x 1.5 x 10 cm and 0.5 x 6 x 10 cm, respectively). Total acrylamide concentration in the resolving gel was 13% and upper reservoir buffer contained 0.1% SDS and 0.2 mM EGTA. All reagents were electrophoresis grade and were purchased from Bio-Rad except for SDS, which was from Pierce.

Results

When the partially purified CaM fraction was applied to a column of melittin-Sepharose in the presence of Ca^{2+}, CaM was selectively retained while contaminating proteins and nucleotides passed through (Fig. 1). The addition of 2 mM EGTA eluted bound CaM, which was homogenous on SDS gel electrophoresis

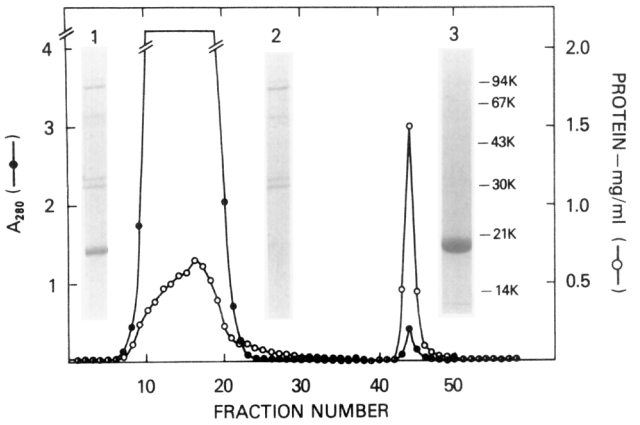

Fig. 1. Purification of CaM by affinity chromatography on melittin-Sepharose. Partially purified CaM (47 mg in 52 ml) was adjusted to 1 mM $CaCl_2$ and applied to a column (2.5 x 8 cm, 40 ml) of melittin-Sepharose (0.6 mg melittin/ml gel) equilibrated in buffer A. After washing with 100 ml of buffer A containing 0.5 M NaCl (final concentration) at a flow rate of 120 ml/h, the column was eluted (flow rate, 60 ml/h) with 100 ml of the buffer A lacking calcium and containing 2 mM EGTA and 10% glycerol (buffer B); this is indicated by the arrow. Fractions (5 ml) were collected and assayed for protein (○) or absorbance at 280 nm (●). SDS gel electrophoresis of the starting material (4 µg), material not retained on the column (2.2 µg), and the EGTA eluate (4 µg) are shown in the inset, lanes 1-3, respectively. Positions of molecular weight standards (Pharmacia) run on the same gel are indicated on the right (phosphorylase a, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21 kDa; α-lactalbumin, 14.4 kDa).

(inset, Fig. 1) and whose ultraviolet absorption spectrum appeared identical to that reported for pure CaM (data provided to reviewers). The ability of CaM to interact with and be eluted from affinity gels was proportional to substituent concentration from 0.6 to 10 mg of melittin/ml of gel, indicating no apparent differences in binding capacity or recovery over this range (data not shown). Under optimal conditions, recovery of CaM from these gels was 0.8-0.9 mg CaM/mg melittin.

The maximal amount of protein bound to a column of melittin-Sepharose containing a total of 3.3 mg melittin was reached with 4 mg (protein) of applied partially purified CaM and did not increase with higher loads (Fig. 2A). Subtraction of the amount of protein not retained from the amount applied for each sample indicated that a maximum of 2 mg protein (using bovine serum albumin as standard) was retained. Analysis by SDS gel electrophoresis (Fig. 2B) showed that, under saturating conditions, this was exclusively CaM. Since the dye-binding method used for measurement of protein (9) underestimates CaM

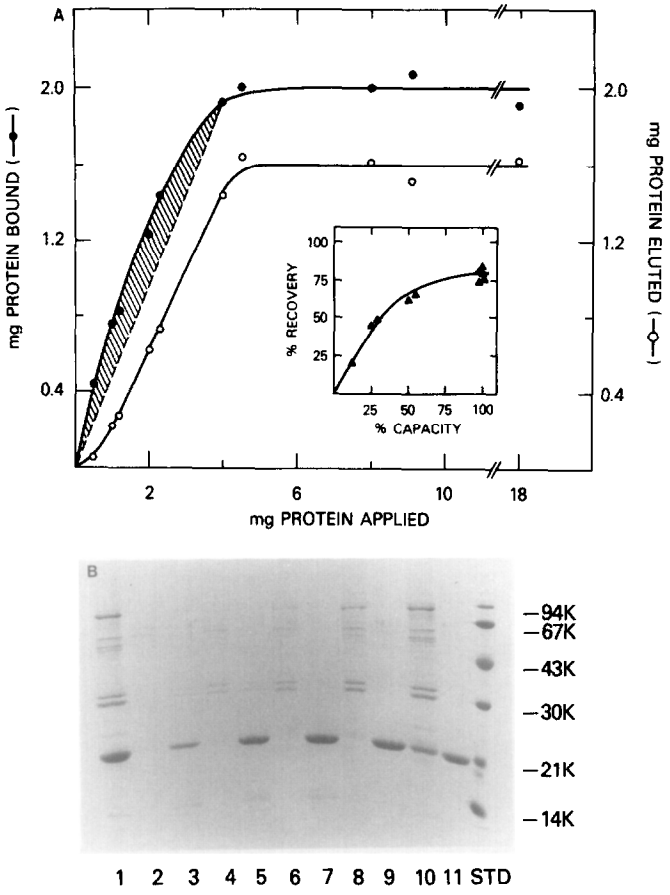

Fig. 2A. Determination of the capacity of melittin-Sepharose for CaM and its relationship to recovery. The indicated amounts of partially purified CaM were applied to columns (0.7 x 1.8 cm, 0.7 ml) of melittin-Sepharose (4.5 mg melittin/ml gel) equilibrated in buffer A, after which columns were washed with four bed volumes of that buffer and eluted with three bed volumes of buffer B (see legend to Fig. 1). The amount of protein not retained was subtracted from that applied; the difference was the amount bound to the column (●). The amount of the protein in the eluate (○) was determined using bovine serum albumin as standard. Since all CaM applied was bound up to 4 mg of applied protein, the dashed line represents the amount of CaM bound at lower amounts of applied protein; the shaded area represents the small amount of non-CaM protein adsorbed at these subsaturating levels. Inset: The percentage of bound CaM recovered in the eluate (ordinate) is plotted as a function of the percentage of column capacity (2 mg protein = 100% capacity).

Fig. 2B. SDS gel electrophoresis of fractions from Fig. 2A. Samples of unretained and eluted fractions (2-4 µg) were precipitated with trichloroacetic acid (10%, w/v) and solubilized in buffer containing 1% SDS prior to electrophoresis (8). Lane 1, 4 µg of starting material; Lanes 2, 4, 6, 8, and 10, material not retained; and Lanes 3, 5, 7, 9, and 11, eluates from samples of 0.5, 1, 2, 4, and 8 mg of applied protein, respectively (see Fig. 2A).

protein by 30-35% when compared to bovine serum albumin, the true capacity for CaM was 2.9-3.0 mg per 3.3 mg melittin or roughly 0.9 mg CaM/mg immobilized melittin. At lower protein loads, there was detectable interaction of non-CaM

protein, a small percentage of which could be eluted with EGTA (Fig. 2B). The major EGTA-eluted component at all amounts of applied protein was, however, CaM. The percentage of bound CaM eluted from melittin-Sepharose varied as a function of the saturation of column capacity. When the amount of CaM bound to the column was less than or equal to 25% of its capacity, recovery was less than 50%. When loads were greater than 60% of the capacity, recoveries approached a maximum of ~ 80%. Inclusion of 0.5 M NaCl in the elution buffer increased maximal recoveries to > 90%, however, the qualitative relationship between column capacity and recovery of bound protein still obtained.

Another Ca^{2+}-binding protein, S-100 protein, also interacted with melittin-Sepharose, although its affinity appeared lower than that of CaM, as judged by displacement of bound S-100 by CaM. (The displacement of this lower M_r peptide by CaM under conditions of column saturation can be observed in Fig. 2B.) When significant amounts of S-100 protein were present in the

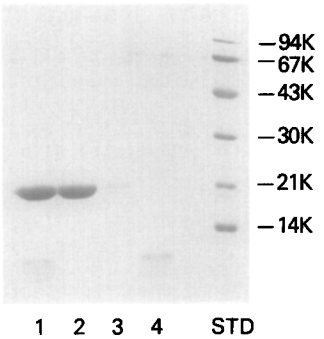

Fig. 3. Separation of CaM and S-100 protein using organomercurial agarose chromatography. For this experiment, partially purified CaM was prepared by a method involving extraction of brain tissue with deionized 6 M urea and batchwise chromatography on QAE-Sephadex A-25 (see Materials and Methods). This fraction was applied to melittin-Sepharose, and the EGTA eluate (5 ml, 5 mg of protein) was applied to a column (0.7 x 3.6 cm, 1.5 ml) of organomercurial agarose (Affi-Gel 501, Bio-Rad). This column was washed with 1.2 bed volumes of buffer B, and the effluent was added to the fraction not retained. After washing with three bed volumes of buffer B, the column was eluted with three bed volumes of buffer B containing 10 mM dithiothreitol. Proteins were precipitated and solubilized for SDS gel electrophoresis as described in the legend to Fig. 2B. Lane 1, 5 µl of starting material; Lane 2, 7 µl of material not retained on organomercurial agarose (CaM); Lane 3, 5 µl of the wash fraction; Lane 4, 5 µl of the eluate (S-100 protein). Positions of molecular weight standards (as in Fig. 1) are shown on the right. Coomassie blue-stained material in the region of 60-70 kDa is a gel artifact since it was found in lanes which contained no protein.

melittin-Sepharose eluate, they were easily removed by adsorption of the S-100 protein to organomercurial agarose. Since CaM contains no free sulfhydryls, it was quantitatively recovered in the unretained fraction while S-100 protein, which often runs as a broad band under conditions of SDS gel electrophoresis, was eluted from this column with thiol (Fig. 3). The sequential use of organomercurial agarose followed by melittin-Sepharose chromatography (see Materials and Methods) permitted the large-scale simultaneous purification of these two Ca^{2+}-binding proteins from bovine, ovine, and porcine brain with yields of > 200 mg/kg tissue in each case.

Since S-100 protein appears to be present in low amount in testis, melittin-Sepharose chromatography alone was sufficient to purify CaM directly from tissue supernatant (Fig. 4), although the apparent capacity (0.4 mg CaM/mg melittin) was lower. This difference is probably attributable to the presence of many lower affinity interactions in crude material which may compete for binding sites.

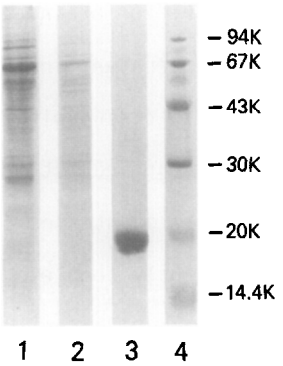

Fig. 4. Single-step purification of CaM from bovine testis supernatant. Thirty grams of bovine testis was homogenized in four volumes of 25 mM BES, pH 7.0, containing 100 mM NaCl, 2.5 $MgCl_2$, 0.5 mM EGTA and PMSF (75 µg/ml). After centrifugation (20,000 x g, 30 min), soybean trypsin inhibitor (20 µg/ml) was added to the supernatant (~ 100 ml) which was made 2 mM with $CaCl_2$ and stirred with 6 ml of melittin-Sepharose (~ 4.5 mg melittin/ml gel) for 2 h. The gel was collected by brief centrifugation, washed with 10 ml buffer A and centrifuged a second time, and poured into a column (diameter 1.5 cm). The gel was washed with four bed volumes of buffer A containing 0.5 M NaCl followed by 0.6 bed volumes of buffer B and, after 30 min, eluted with three bed volumes of buffer B (yield ~ 8 mg CaM) SDS gel electrophoresis of supernatant, 6 µg (Lane 1), wash fraction, 2 µg (Lane 2), melittin-Sepharose eluate, 4 µg (Lane 3), and protein standards (Lane 4) as in Fig. 1.

Discussion

Calmodulin has been purified to homogeneity from numerous tissues and species using conventional chromatography methods, e.g., ion-exchange and gel filtration (10-12), as well as affinity chromatography (13,14). The latter methods take advantage of the interaction of small hydrophobic ligands such as phenothiazine or phenyl groups immobilized on agarose with a hydrophobic region of CaM that is exposed when Ca^{2+} is bound (15,16). Although highly enriched in CaM, fractions prepared with these latter methods often require additional chromatographic steps to achieve homogeneity. Brain contains another acidic, Ca^{2+}-binding protein, S-100 protein, that may contaminate such preparations. Indeed, S-100 protein also interacts strongly with hydrophobic ligands and its purification has been achieved using an immobilized naphthalene sulfonamide derivative (17).

We have exploited the high affinity interaction between melittin and CaM to prepare a very high capacity affinity matrix which exhibits low nonspecific interactions. In fact, the amount of contaminants eluted with CaM appears to be negligible under appropriate loading and washing conditions.

The method has proven successful for brain and for testis with a one-step purification directly from crude supernatant in the latter case. For highest yield and purity, extraction of brain tissue with urea followed by batchwise absorption to ion-exchange media is carried out prior to melittin-Sepharose chromatography. Purification of gram quantities of CaM from 4-12 kg brain based on this method can be conveniently completed in two days with average yields of 250-300 mg/kg tissue. Homogenization of tissue in the presence of 6 M urea (18) solubilizes a large fraction of CaM which is otherwise difficult to extract (~ 40%). The subsequent batchwise use of QAE-Sephadex A-25 provides in addition to anion-exchange chromatography a selective concentration of smaller proteins and nucleotide; the eluate from this gel shows essentially only CaM and S-100 protein with minor amounts of higher molecular weight peptides. Since CaM does not contain free sulfhydryl groups, it does not bind to organomercurial agarose which can be used to remove non-CaM protein from

the fraction obtained by ion-exchange chromatography. After elution from organomercurial agarose, S-100 protein can be purified on melittin-Sepharose in yields comparable to those of CaM (~ 200-250 mg/kg tissue). No substantial changes in capacity were noted after repeated use and regeneration of the matrix with 6 M urea, suggesting that the properties of this column are stable even with crude starting materials.

The proportionality between column capacity and substituent concentration over a 15-fold range of bound ligand indicates that melittin, which can exist as either monomer or tetramer (19), must be immobilized to the column in its monomeric form, since at protein concentrations of 2 mg/ml and greater melittin would exist largely as tetramer. The capacity of the affinity matrix, ~ 1 mg CaM/mg melittin, which is constant with repeated use, indicates that approximately one out of six melittin molecules is capable of high affinity, reversible interactions with CaM. An interesting correlation was that the recovery of bound CaM depended on the degree of column occupancy. Although recoveries were 85-95% under saturating conditions, when less than 50% of the operational capacity was utilized, recovery of bound CaM decreased. In practical terms, saturation of column capacity is important not only for recovery but appears to minimize binding of weaker interacting species, such as S-100 protein.

In addition to its value as a large-scale purification medium, melittin-Sepharose may prove useful for other biochemical applications. Recently, binding of CaM to this matrix has allowed selective chemical modification of the protein. After elution from this support, further modification of sites, presumably protected at the interaction domain, has been carried out (Kincaid, R., unpublished data). Thus, it may be possible to probe the specific regions involved in protein-protein interaction using such chromatographic approaches.

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