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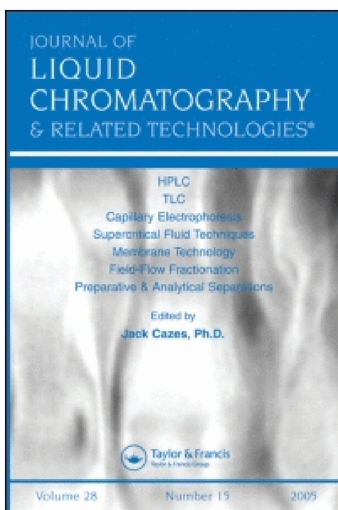

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Separation of Tyrosine Phosphorylated Calmodulin from Calmodulin Using Non-Porous Anion-Exchange HPLC

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SEPARATION OF TYROSINE PHOSPHORYLATED CALMODULIN FROM CALMODULIN USING NON-POROUS ANION-EXCHANGE HPLC

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ABSTRACT

Calmodulin is a protein which exerts control over various physiological processes by binding and modulating key enzymes after it undergoes a calcium-induced conformational change. The insulin receptor kinase has been shown to phosphorylate tyrosine 99, in the third calcium binding cleft of the protein. Since each molecule accommodates only four calcium ions, the introduction of phosphate into one of these calcium binding pockets may alter biological activity. To determine the effects of this modification, required the separation of phosphorylated calmodulin from the unphosphorylated form.

This paper addresses the HPLC techniques we have used for the successful separation of various forms of calmodulin. Reversed-phase HPLC at pH 7.8 in the presence of 100µM $CaCl_2$ or 1mM EGTA gave different elution profiles, reflecting the effect Ca^{++} has on calmodulin conformation. Such changes were not observed using 0.1% trifluoroacetic acid in mobile phases since calmodulin binds essentially no calcium at pH 2.0. Anion-exchange HPLC on a non-porous DEAE-type resin was found to be superior to reversed-phase methods for the specific separation of phosphorylated calmodulin from unphosphorylated calmodulin.

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INTRODUCTION

Calmodulin is a protein which appears to exist in all eukaryotic cells (1). This protein binds four moles of calcium per mole protein and undergoes a conformational change upon calcium binding. By a direct route, calmodulin binds to enzymes such as cyclic nucleotide phosphodiesterase (2), myosin light chain kinase (3), (Ca^{++} + Mg^{++})-ATPase (4, 5) and in the presence of Ca^{++} activates or enhances their activites. We recently described phosphorylation of calmodulin by the insulin receptor kinase both by solubilized receptor preparations and in intact cells (6-8). Other groups have shown that calmodulin can serve as a substrate for phosphorylation by other kinases (9-12). Protein phosphorylation is involved in numerous cellular processes including the regulation of protein synthesis, various metabolic pathways, and the signal transduction of transmembrane and mitogenic events (13). Most proteins are phosphorylated to some degree, but the functional significance of this modification is often unclear. An intriguing possibility is that phosphorylation of calmodulin may affect some of the modulatory roles described above. To our knowledge, this question has previously not been addressed because a procedure for the separation of phosphorylated calmodulin from the non-phosphorylated form has not been available.

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We have developed an high-performance liquid chromatography (HPLC) method to separate calmodulin which is phosphorylated at tyrosine 99 by the insulin receptor-associated tyrosine kinase from the unphosphorylated protein. Since tyrosine 99 resides in the third calcium binding cleft of calmodulin, we hypothesize that this modification may alter calcium interaction at this position. Therefore, the calcium-binding properties of calmodulin were considered in the separation by adjusting the amount of calcium ion in the HPLC buffers. In the past, this calcium-binding characteristic has been utilized (14, 15) or avoided (16) by researchers employing chromatography to isolate calmodulin or calmodulin fragments. We examined various buffer systems of reversed-phase and anion-exchange HPLC; under these

conditions only anion-exchange on a non-porous diethylamino ethyl (DEAE) resin could resolve phosphocalmodulin from calmodulin.

MATERIALS AND METHODS

All solvents were HPLC grade from Burdick and Jackson (Muskegon, MI). Trifluoroacetic acid was purchased from the Pierce Chemical Co. (Rockford, IL) and purified over a bed of alumina before use (17). Sodium phosphate, Trizma base and Tris-HCl were purchased from the Sigma Chemical Co. (St. Louis, MO). All buffers were filtered through .45um filters (Millipore, Milford, MA).

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High Performance Liquid Chromatography

HPLC was performed using a Varian 5000 liquid chromatograph (Walnut Creek, CA) with a Hitachi L-3000 diode array detector (EM Science, Cherry Hill, NJ), Kratos 773 detector (Applied Biosystems, Foster City, CA) or Varian UV-100 detector. The Vydac C_4 (4.6mm x 25cm) reversed-phase column was purchased from The Separations Group (Hesperia, CA). The Bio-Gel TSK Phenyl 5PW (7.5mm x 7.5cm) and Bio-Gel TSK DEAE 5PW (7.5mm x 7.5cm) columns were obtained from Bio-Rad (Richmond, CA). The Progel™ TSK NPR DEAE (4.6mm x 3.5cm) column was a gift from Supelco, Inc. (Bellefonte, PA). Chromatography was performed at room temperature with fractions collected at one minute intervals. Since the calcium-bound form of calmodulin is difficult to elute under hydrophobic interaction chromatography (HIC) conditions we used the TSK phenyl column in the reversed-phase mode (18). Retention times for calmodulin on the various HPLC columns were determined by both elution time of standard and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of fractions. Phosphocalmodulin containing fractions were monitored by detection of Cerenkov radiation and confirmed by SDS-PAGE and autoradiography.

Phosphocalmodulin Preparation

Standard bovine brain calmodulin was purchased from United States Biochemical Corp. (Cleveland, OH) or Ocean Biologics (Edmonds,WA). The calmodulin phosphorylated *in vitro* by solubilized insulin receptor-associated tyrosine kinase was prepared by a method previously described (6, 7). The cell-free extract was first solid phase extracted on C-18 Sep-Paks (Waters Assoc., Milford, MA) before any mode of HPLC was performed. To achieve the desired HPLC separation we were seeking it was necessary to resolve the unlabeled calmodulin (~95% of the mass) from the much smaller amount of labelled (~5%) phosphorylated calmodulin.

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Electrophoresis

Electrophoresis was performed by a method already described (19) for routine confirmation of calmodulin and phosphocalmodulin-containing chromatography fractions. In short, a 9-26% SDS-PAGE gradient slab gel technique which greatly expands the molecular weight separation region in the 5kD to 25kD range (20, 21) was used. Coomassie stained gels were dried and exposed to Kodak XAR-5 film (Rochester, NY) to identify ^{32}P-labelled phosphocalmodulin.

RESULTS AND DISCUSSION

When calmodulin binds calcium, it undergoes conformational changes which expose hydrophobic region(s) and enable it to fill binding sites on target enzymes (2-5). This characteristic has been used to purify calmodulin by affinity chromatography and HIC (14, 15). In the presence of trace amounts of calcium in the mobile phase, calmodulin can be separated into calcium-bound and calcium-free states by reversed-phase HPLC (16). Previously, we used the 0.1% TFA/acetonitrile reversed-phase method to partially purify

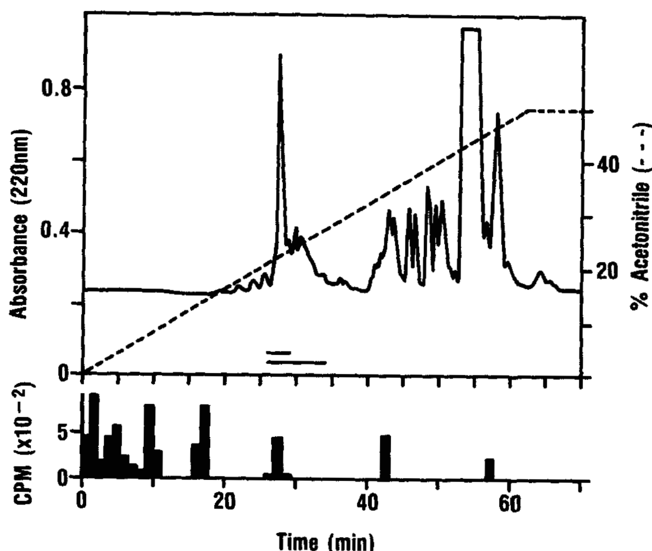

FIGURE 1. Reversed-phase HPLC at pH 7.8 of phosphorylated material from cell-free incubation (see materials and methods) is shown. The Vydac C_4 column (4.6mm x 25cm) was run at a flow rate of 1ml/min with a gradient of 0 to 100% B in 60min. Buffer A was 35mM ammonium bicarbonate and buffer B was 50% acetonitrile in buffer A. Both buffers contained 100µM $CaCl_2$. Cerenkov radiation in the fractions is shown below the chromatogram. Phosphorylated calmodulin (counts) eluted from 27-29 minutes and non-labeled calmodulin eluted from 27 through 34 minutes.

calmodulin phosphorylated by insulin in adipocyte membrane extracts (6). However, the pH of 0.1% TFA is approximately 2.0 and calmodulin shows little affinity for Ca^{++} below a pH of 3.5 (22). Although the calmodulin peak was sharp with this system, phosphocalmodulin co-eluted with unmodified calmodulin and therefore other systems needed to be explored. Reversed-phase at pH 7.8 was attempted as one alternative. In the presence of 100µM calcium, four to five non-phosphorylated calmodulin peaks were observed that were not baseline resolved. Phosphocalmodulin comigrated with the mass peak in this system (Fig. 1). This profile collapsed to one sharp phosphocalmodulin/calmodulin peak which eluted 4 minutes later

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when 1mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) was added to both buffers (data not shown). Although various conformers of calmodulin could be separated with the pH 7.8 reversed-phase system in the presence of calcium, specific isolation of phosphotyrosyl-calmodulin away from all non-phosphorylated forms could not be achieved.

Two peaks were seen when commercially obtained calmodulin was eluted from a phenyl column with calcium-containing buffers (Fig. 2a). Interestingly, isolated ^{32}P-phosphorylated calmodulin was found to elute in both of these time frames (Fig. 2b). This method was not suitable for the intended separation of phosphocalmodulin from calmodulin, but did suggest that phosphocalmodulin may undergo a conformational change similar to non-phosphorylated calmodulin.

Fig. 3 illustrates the profile generated when the phosphocalmodulin preparation was applied to a TSK DEAE porous polymer anion-exchange resin. SDS-PAGE and autoradiography were performed on all chromatography fractions for molecular mass identifications (data not shown). The order of eluted radioactive pools from this cell free extract was autophosphorylated insulin receptor kinase (13-15 min), followed by an unidentified 3 kilodalton peptide (24-26 min) and finally calmodulin (38-40 min). The radioactivity corresponding to the calmodulin peak (minutes 38-40, lower panel) corresponds directly to the major absorbance peak (upper panel), indicating that resolution of calmodulin from phosphocalmodulin was not achieved. The top panel of Fig. 3 shows that the stainable mass of protein aligned directly with the autoradiography SDS-PAGE bands. Since the ratio of non-phosphorylated to phosphorylated calmodulin was approximately 20:1 in this preparation, complete resolution of phosphorylated calmodulin would be indicated by separation of radioactive material from the majority of protein. The addition of 100μM calcium to the anion-exchange mobile phases did not significantly change the elution profile except for slightly decreasing overall retention times.

In contrast to the results with the porous resin, when the same preparation of partially phosphorylated calmodulin was applied to a

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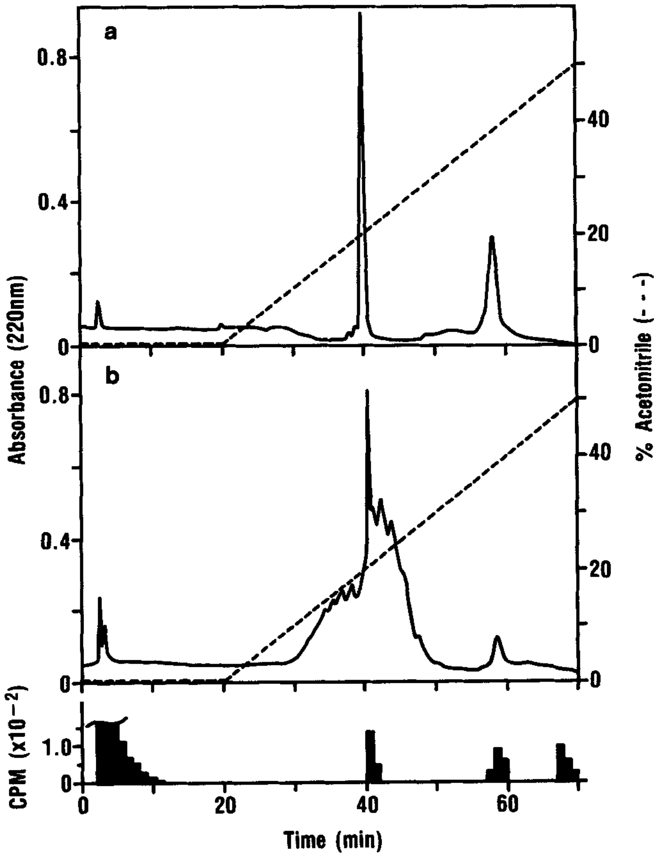

FIGURE 2. Reversed-phase HPLC of (a) standard bovine brain calmodulin and (b) phosphorylated sample identical to that shown for Fig. 1 were chromatographed on a TSK phenyl 5PW (7.5mm x 7.5cm) column in sodium phosphate buffer (10mM, pH-4.0) with 0 to 50% gradient of acetonitrile. Two fractions of radioactivity in (b) (minutes 40-42 and 57-60) were shown by SDS-PAGE and autoradiography to be phosphocalmodulin. The radioactivity in minutes 63-65 corresponded to a molecular mass too small (< 1.5 kD) for SDS-PAGE analysis.

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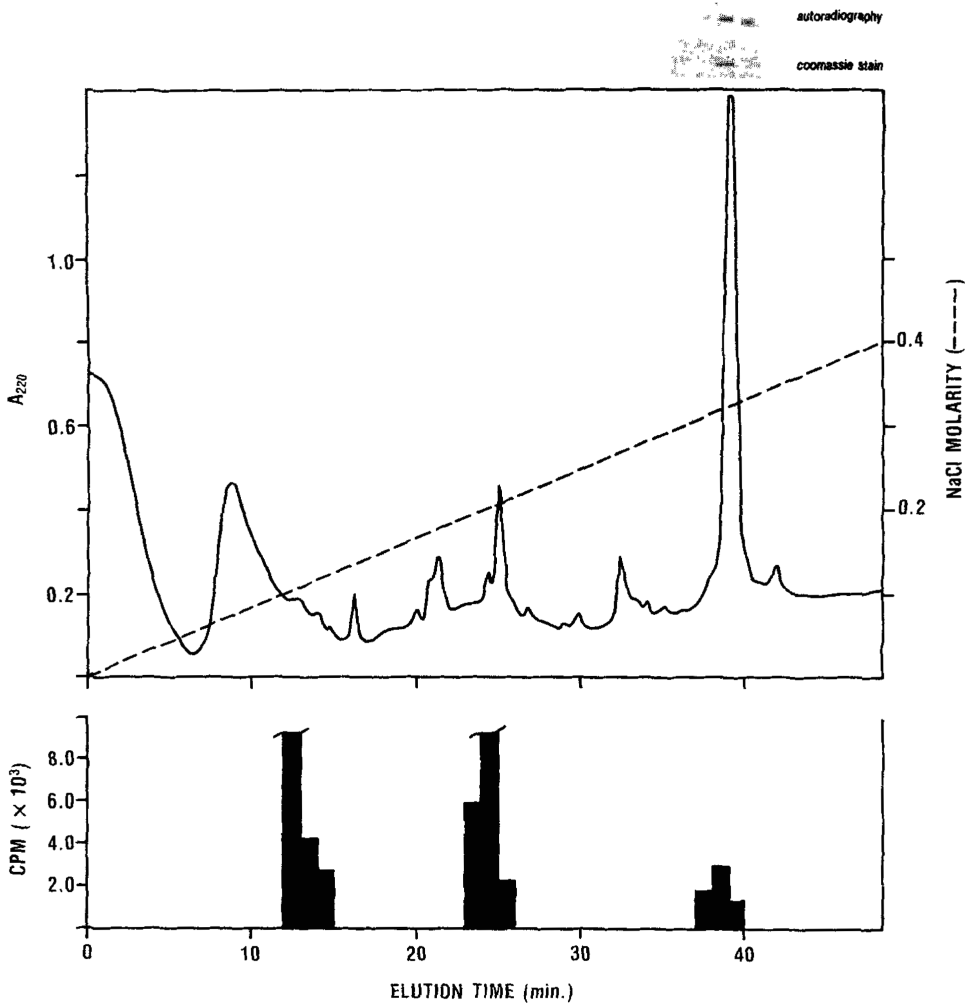

FIGURE 3. Anion-exchange HPLC with a TSK (porous) DEAE 5PW (7.5mm x 7.5cm) column at a flow rate of 1.0ml/min is shown. The sample was as described in Fig. 1. The gradient was 0 to 0.5M NaCl in 60min in 20 mM Tris (pH 8.0) buffer. Fractions from this chromatogram were counted for Cerenkov radiation (lower panel) and subjected to SDS-PAGE and autoradiography (top panel above chromatograph).

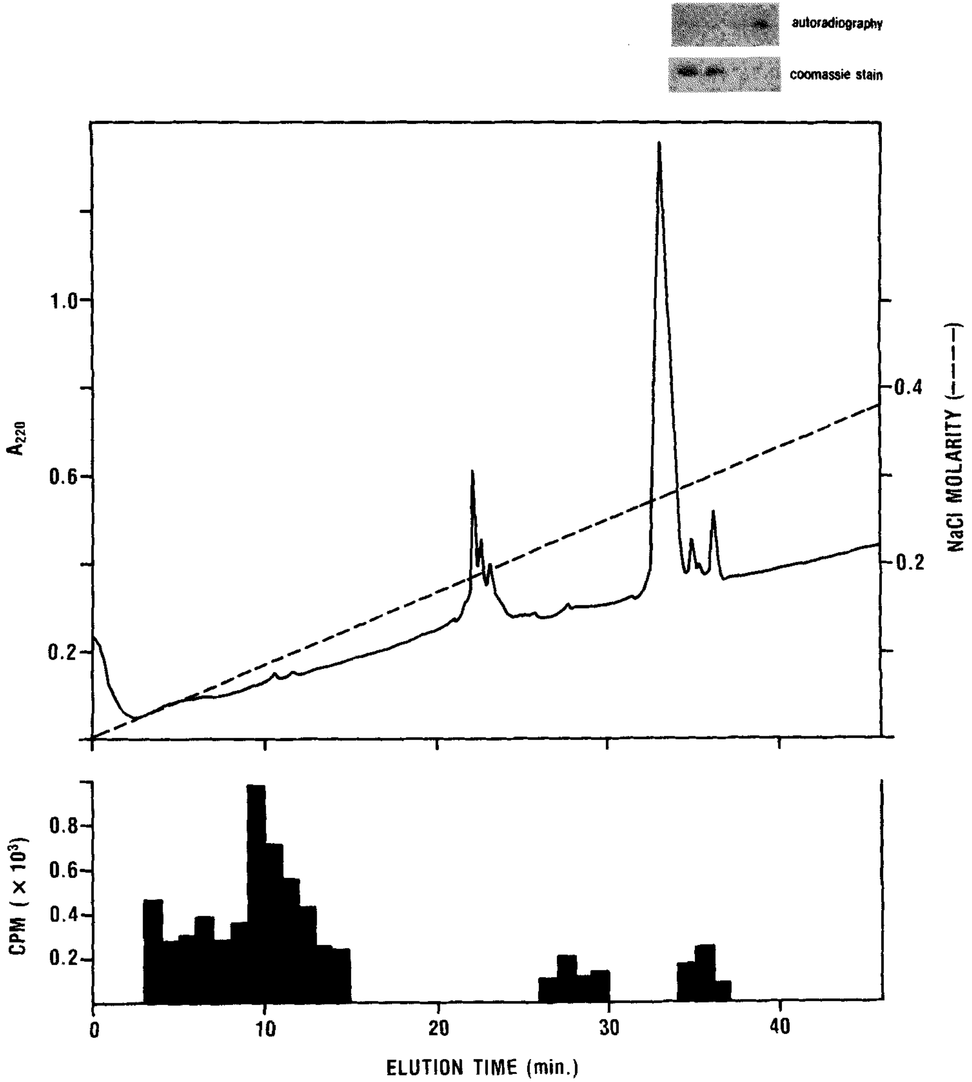

FIGURE 4. Anion-exchange separation of phosphorylated calmodulin from non-phosphorylated calmodulin on a TSK NPR (non-porous) DEAE column (4.6mm x 3.5cm). Gradient, buffer system, and flow rate were identical as in Fig. 3. The Cerenkov radiation (lower panel), and SDS-PAGE bands (top) are shown as in Fig. 3.

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TSK non-porous resin DEAE column, phosphorylated calmodulin was clearly separated from the majority of protein mass (Fig. 4). The phosphorylated calmodulin (radioactivity) peak eluted at 34-36 minutes and was, clearly resolved from the major mass peak of unphosphorylated calmodulin which eluted at 32-33 minutes. SDS-PAGE analysis of the calmodulin HPLC fractions in the top panel of Fig. 4 confirmed the spectral observation. The radioactivity between 27-30 minutes was due to unidentified 3kD phosphopeptide material present in the reaction mixture (not shown). Peak shaving enabled us to collect two essentially homogeneous pools corresponding to the tyrosine phosphorylated and unphosphorylated calmodulin.

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CONCLUSION

Reversed-phase HPLC with and without calcium in the mobile phases did not fully resolve phosphorylated calmodulin from the unphosphorylated protein. These studies included low (~2.0) and high (7.8) pH systems on C_4 and phenyl columns. In contrast, anion-exchange HPLC was fully effective at resolving phosphorylated calmodulin from the unphosphorylated form. However, the column support matrix as well as DEAE selectivity were important in this regard. Under these conditions, the non-porous particle DEAE column was effective while the column containing porous hydrophilic polymer coated with DEAE was ineffective for this separation. The selectivity of this non-porous based resin for proteins may be beneficial in other difficult separations in which constituents differ only by a phosphate group.

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