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Isolation of calcium-binding proteins on selective adsorbents Application to purification of bovine calmodulin

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Abstract

We report the fractionation of calcium-binding proteins using immobilized metal ion affinity chromatography (IMAC) with hard metal ions. Various hard metal ions (Mn^{2+}, La^{3+}, Nd^{3+}, Eu^{3+}) were immobilized on cross-linked agarose substituted with Tris(carboxymethyl)ethylenediamine (TED) and used as an adsorbent. After systematic studies, europium was selected for further work on the fractionation of calcium-binding proteins. It was found that the presence of Ca^{2+} in the sample and the solvent strongly promoted the adsorption and selectivity. Selective elution was accomplished in stepwise mode by the addition of calcium chelators such as malonate, citrate and phosphate. Calmodulin of high purity was isolated from a crude extract. Similar behavior of other calcium-binding proteins indicates that the reported chromatographic procedure can be generally applied to such proteins.

Keywords: Calmodulin; Proteins; Calcium-binding proteins; IMAC

1. Introduction

In 1975 Porath et al. introduced immobilized metal ion affinity chromatography (IMAC) (at that time called metal chelate chromatography) using the 3-d elements zinc and copper [1]. As applied to the "intermediate" type metal ions IMAC has met with considerable success [2,3]. The main electron donor target on the proteins for the "intermediate" metal ions is the imidazole nitrogen of the histidine side chain [4,5]. It is desirable to extend IMAC to the purification of proteins with other surface-located electron donors. Hard metal ions, such as calcium, have a preference for oxygen and the calcium-binding proteins are, therefore, potential objects for extension of IMAC principle in the direction of hard metal ions.

Some pioneering work has already been done in the field [6–10] in Porath's laboratory. However, twenty years after the introduction of IMAC, we are still not able to exploit beneficially the affinity of numerous biological molecules towards the most abundant metal ion in the living matter for their purification and/or immobilization.

One obstacle encountered in designing an IMAC procedure for calcium-binding proteins concerns the strength of binding of calcium to the support. It is not easy, in fact for most purposes, it is impossible to find ligands that will bind calcium strongly enough to match the affinity of the calcium-binding proteins for the metal ions. Calcium ions are desorbed from

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the adsorbent and transferred to the proteins (so-called metal ion transfer (MIT)) [11].

In an attempt to solve this problem, we have substituted calcium with other hard metal ions, such as lanthanides. The lanthanides, Mg(II) and Mn(II), were reported as substitutes of Ca(II) in regulation of Ca(II) dependent proteins, as well as spectroscopic and X-ray probes [12–18]. We have found that the presence of calcium ions in the equilibration buffer enhances the capacity and the selectivity of such adsorbents.

We demonstrate the potential of IMAC with lanthanides to selectively adsorb calcium-binding proteins using calmodulin (CaM) as a model protein after an initial purification step with IMAC on Fe(III)–IDA–agarose [19]. It appears that these methods can be extended to other calcium-binding proteins as well.

2. Materials and methods

2.1. Materials

Novarose TED^{High}-1000/40 (with capacity of 65 μmol Cu(II)/ml gel) was kindly provided by Inovata AB (Stockholm, Sweden). PIPES (Piperazine-N,N′-bis[2-ethanesulfonic acid]), Tris (Trizma® Base), citrate, malate, malonate, oxalate, tartrate and imidazole were from Sigma (St. Louis, MO, USA). $CaCl_3$, $EuCl_3$, $FeCl_3$, $LaCl_3$ and $NdCl_3$ were from Aldrich Chemical (Milwaukee, WI, USA). All chemicals used were of chemical grade or higher. Albumin and IgG were obtained from Sigma (St. Louis, MO, USA).

CaM was isolated from bovine testes by homogenizing and extracting the tissue, followed by purification and concentration by chromatography on DEAE–Sepharose FF and hydrophobic interaction chromatography according to Autric et al. [20] and Gopala Krishna et al. [21]. It is here labeled "purified CaM". The desorbed material from DEAE–Sepharose was freeze-dried. It is here labeled "crude extract".

2.2. Preliminary screening experiments

The purified CaM was dissolved in the respective starting buffer for IMAC and used directly as a starting sample. The crude extract of CaM was dissolved in the respective starting buffer for IMAC and the resulting suspension was centrifuged for 20 min at 14 000 rpm and 10°C. The clear supernatant was equilibrated in PD-10 columns (Pharmacia Biotech) against the respective starting buffer and used as a starting sample for IMAC. Novarose TED^{High}-1000/40 packed in columns (9 cm×0.5 cm I.D.) was charged with Ca(II), La(III), Nd(III), Mn(II) and Eu(III) ions by applying five column volumes of 20 m*M* solutions of the salts of each metal ion, followed by washing with five column volumes of deionized water and equilibration with the respective starting buffer. The equilibrated sample containing CaM was loaded on the columns and the non-adsorbed material was removed with the respective starting buffer. The elution was performed as described in the Results section.

2.3. Frontal analyses

Frontal analyses of purified CaM on metal-free and charged with Ca(II) and Ca-substituting metal ions Novarose TED^{High}-1000/40 were performed with 1 mg/ml CaM in the starting buffer (0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5). The eluent volume, V_m, at which 0.5 mg/ml CaM concentration was detected was used to determine the capacity of the adsorbent [22].

2.4. Purification of CaM from crude extract

2.4.1. Step 1. IMAC on Fe(III)–IDA–agarose

The IMAC column was prepared as follows: Chelating Sepharose FF was packed in a column (6.3 cm×1.0 cm I.D.) and charged with Fe(III) ions by applying five column volumes of 20 m*M* $FeCl_3$. The excess Fe(III) was removed by washing with five column volumes of water. The column was equilibrated with 20 m*M* PIPES, 1.0 *M* NaCl pH 6.1. Crude extract (300 mg) was dissolved in 6 ml of 20 m*M* PIPES, 1.0 *M* NaCl pH 6.1. The solution was further equilibrated to the same buffer on a PD-10 (Sephadex G-25) column and used as a starting sample. After loading and washing with the equilibration buffer, CaM was eluted by increasing the pH to 6.9 (20 m*M* PIPES, 1.0 *M* NaCl). The

eluted fraction served as a starting sample in the second step.

2.4.2. Step 2: IMAC on Eu(III)–TED–agarose

Novarose TED^{High}-1000/40 packed in a column (6.3 cm×1.0 cm I.D.) was charged with Eu(III) ions by applying five column volumes of 20 mM $EuCl_3$. The excess Eu(III) was removed by washing with five column volumes of water. The column was equilibrated with 0.1 *M* Tris–HCl, 2.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5. After loading the sample and washing with the same buffer for removal of the non-adsorbed material, the column was washed with 0.6 *M* Na_2SO_4 in 0.1 *M* Tris–HCl, 2.0 *M* NaCl pH 7.5. Additional impurities were removed with 40 m*M* malonate in 0.1 *M* Tris–HCl, 2.0 *M* NaCl, 0.6 *M* Na_2SO_4 pH 7.5. CaM was eluted with 0.2 *M* potassium phosphate, 3.0 *M* NaCl, 0.2 *M* citrate pH 7.5. The column was regenerated with 0.1 *M* EDTA pH 7.0.

2.5. Protein analyses

The protein content was determined by the method of Bradford [23] with bovine serum albumin as a standard for protein mixtures and CaM as a standard for the experiments carried out with purified CaM. The concentration of extensively purified CaM was determined also by using a coefficient of 0.27 AU at 280 nm for 1 mg/ml. The recovery and capacity of the gels were determined by spectroscopy using the coefficient given above. The purification factors were determined by using UV spectroscopic analyses (where 1 AU at 280 nm corresponded to 1 mg/ml for the crude extract and 0.27 AU at 280 nm corresponded to 1 mg/ml protein of the extensively purified CaM) as well as by the method of Bradford as described above.

The electrophoretic analyses were performed with Phast System (Pharmacia) on PhastGel Gradient 8–25 gels using PhastGel SDS Buffer Strips for SDS–electrophoresis or Phast IEF 3–9 gels for IEF. PhastGel Blue R was used for visualization of the protein bands. The identification of CaM in electrophoretic gels was made by running the purified CaM as a standard.

3. Results

3.1. Determination of suitable adsorption conditions

Immobilized Eu(III) was used to determine the optimal starting conditions for adsorption of CaM. The optimal pH for CaM loading was determined by running a set of experiments at pH 5.5 and 7.5 with different types of protein-structure formation additives (Fig. 1). As a control the binding of albumin and IgG was followed under the same conditions.

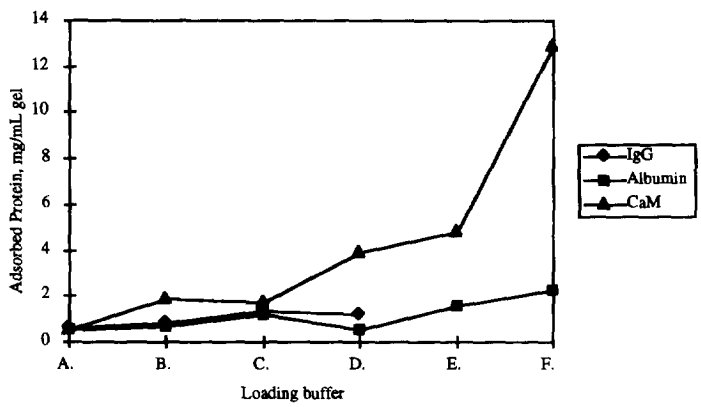

Fig. 1. Adsorption capacity of selected proteins on Eu(III)–TED–agarose in the buffer systems A–F. The equilibration buffers are as follows: A, 100 m*M* MES, 0.8 *M* Na_2SO_4 pH 5.5. B, 100 m*M* Tris–HCl, 0.8 *M* Na_2SO_4 pH 7.5. C, 100 m*M* Tris–HCl, 3.0 *M* NaCl pH 7.5. D, 100 m*M* Tris–HCl, 2.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5. E, 100 m*M* Tris–HCl, 3.0 *M* NaCl, 40 m*M* $CaCl_2$ pH 7.5. F, 100 m*M* Tris–HCl, 3.0 *M* NaCl, 200 m*M* $CaCl_2$ pH 7.5. The loading of the protein was interrupted when a protein breakthrough was detected. The capacity was determined on a basis of the desorbed material.

The protein loading was interrupted when a protein breakthrough was detected. The amount of the adsorbed material was used to determine the capacity.

The capacity of the adsorbent increases with the increase of pH which indicates a binding mechanism that is different from that on immobilized Fe(III). The latter shows higher adsorption capacity below pH 7.0 [19].

NaCl, as well as $CaCl_2$, is required to obtain selective adsorption and high capacity for CaM. The ratio between the adsorbed CaM and non-specifically bound albumin increases from 1 to 5.6 when 200 m*M* $CaCl_2$ is added and pH is increased from 5.5 to 7.5. It would be interesting to find out whether further increases of both NaCl and $CaCl_2$ would result in even higher capacity. The influence of $CaCl_2$, was unexpected (it exceeds the cellular concentration by many orders of magnitude). We chose to use NaCl because of the wide range of concentrations in which it can be used.

The experiments performed in absence of salts (not shown) or in presence of Na_2SO_4 (Fig. 1) resulted in adsorption of no or very little (0.50–0.55 mg/ml gel) CaM. These experiments corroborate those published by Mantovaara et al. [8].

3.2. Optimization of the elution

Various organic acids were tested for their ability to elute the adsorbed CaM from Eu(III)–Novarose TED^{High}-1000/40. Among those were malate, malonate, oxalate, tartrate and citrate, known to form chelates with calcium. Malonate and tartrate were not effective at all for elution of the adsorbed CaM. Oxalate is insoluble in 3.0 *M* NaCl. When tested it

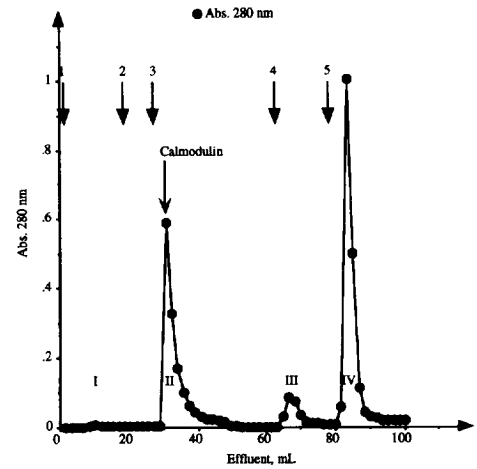

Fig. 2. Chromatogram of extensively purified commercial CaM on Eu(III)–TED–agarose column. Sample: 12.5 mg of CaM dissolved in 4 ml of 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5. Column: 9.5×0.5 cm I.D. (1.865 ml) Eu(III)–Novarose TED^{High}-1000/40 equilibrated with 5 column volumes of 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5. Chromatography: Flow-rate, 0.17 ml/min. 1: Loading of the sample and washing with 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5. 2: Washing with 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.6 *M* Na_2SO_4 pH 7.5. 3: Elution with 0.2 *M* K_2HPO_4, 3.0 *M* NaCl, 0.2 *M* citrate pH 7.1. 4: Elution with water. 5: Elution with 0.1 *M* EDTA pH 7.0.

was found that only citrate and malonate were suitable displacers. In addition, imidazole and phosphate were tested for their ability to elute CaM. The results of these experiments are presented in Table 1.

The finding that imidazole has no effect on binding indicates that the binding mechanism is different from that of the transition metal ions. It was somewhat surprising to see that IDA has only a moderate elution effect. The best eluting buffer is composed from equimolar amounts (0.2 *M*) of K_2HPO_4 and citrate (Peak II in Fig. 2). In all cases

Table 1
Optimization of elution

Elution buffer	Recovery, % of adsorbed CaM
0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* imidazole pH 7.5	0
0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* IDA pH 7.5	25
0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* malate pH 7.5	28
0.3 *M* K_2HPO_4, 3.0 *M* NaCl pH 7.0	68
0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* citrate pH 7.5	73
0.1 *M* K_2HPO_4, 3.0 *M* NaCl, 0.2 *M* citrate pH 7.0	84
0.1 *M* K_2HPO_4, 3.0 *M* NaCl, 0.4 *M* citrate pH 7.0	85
0.2 *M* K_2HPO_4, 3.0 *M* NaCl, 0.2 *M* citrate pH 7.0	89

the rest of the adsorbed CaM was completely recovered by removal of the salt from the buffer (Peak III in Fig. 2). The regeneration of the column with 0.1 *M* EDTA pH 7.0 resulted in fractions with high UV absorbance at 280 nm that did not contain any protein (Peak IV in Fig. 2). Peak I in Fig. 2 contained a small amount of impurities present in the purified CaM.

3.3. Frontal analyses

A number of lanthanides, as well as Mn(II), were investigated for their ability to substitute Ca(II) in the adsorption of CaM (Fig. 3). Although La(III) showed highest capacity, it was not possible to desorb CaM with any of the selected elution buffers. Buffers without water-structuring salts (such as Na_2SO_4) could not accomplish complete elution of CaM. Thus, about 10% of the adsorbed CaM was found in the fractions eluted during the regeneration with 0.1 *M* EDTA pH 7.0. These results indicate very strong protein–metal ion interactions that must be avoided; this is the reason why we selected Eu(III) ions for our further studies.

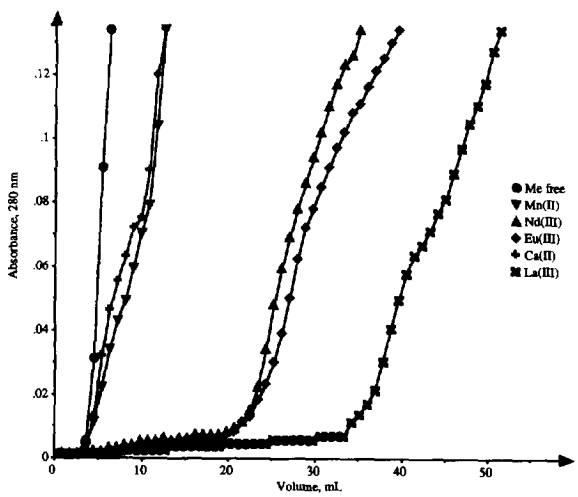

Fig. 3. Frontal chromatograms of extensively purified commercial CaM on metal-free and charged with metal TED–agarose. In each experiment a solution of CaM with a concentration of 1 mg/ml in 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5 was loaded continuously into a column (9 cm×0.5 cm I.D.) at a flow-rate of 0.17 ml/min (the metal-free experiment was performed in the absence of $CaCl_2$).

3.4. Purification of CaM from crude extract

3.4.1. Preliminary purification study

The optimized loading and elution conditions were applied to a crude CaM extract from bovine testes. The chromatography resulted in only five-fold purification (Fig. 4). The majority of CaM together with other proteins present in the extract was detected in Peak III (not shown). Traces of CaM were observed in Peak IV (not shown). Peaks I, II and V did not contain CaM (not shown). It was obvious that a preliminary purification step, as well as optimization of the loading and elution conditions were required to achieve higher purity.

3.4.2. Optimized purification procedure

Step 1: IMAC on immobilized Fe(III) was introduced as a preliminary step (Fig. 5), before the last step on immobilized Eu(III). The selection of the conditions is based on earlier experience with Fe(III) [22,24]. CaM was quantitatively eluted from the

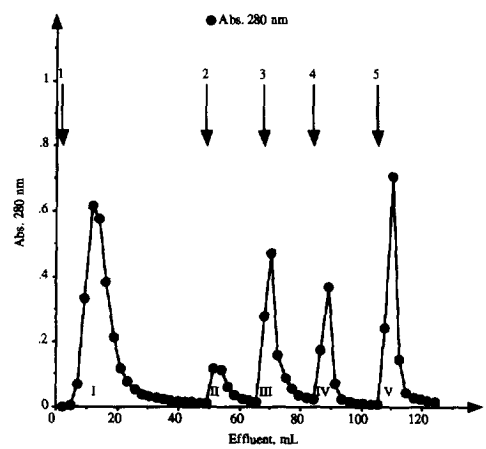

Fig. 4. Chromatogram of a crude extract containing CaM on Eu(III)–TED–agarose. Sample: 50 mg of crude CaM dissolved in 6 ml of 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5. The suspension was centrifuged for 5 min in Eppendorf tubes. Column: 9.5×0.5 cm I.D. (1.865 ml) Eu(III)–Novarose TED^{High}-1000/40 was equilibrated with 5 column volumes of 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 3.0 *M* $CaCl_2$ pH 7.5. Chromatography: Flow-rate, 0.17 ml/min. 1: Loading of the sample and washing with 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5. 2: Washing with 0.1 *M* Tris–HCl, 3.0 *M* NaCl, 0.6 *M* Na_2SO_4 pH 7.5. 3: Elution with 0.2 *M* K_2HPO_4, 3.0 *M* NaCl, 0.2 *M* citrate pH 7.5. 4: Elution with water. 5: Elution with 0.1 *M* EDTA pH 7.0.

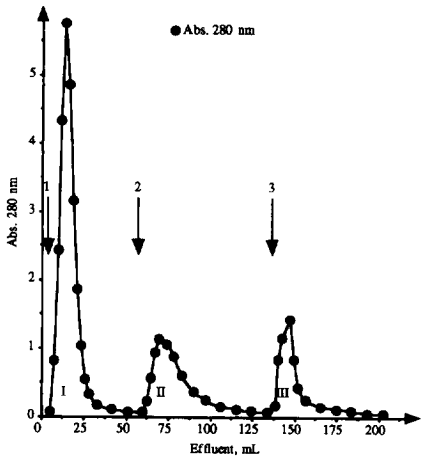

Fig. 5. Chromatogram of crude CaM on Fe(III)–IDA–agarose. Sample: 300 mg of crude CaM is dissolved in 6 ml of 20 m*M* PIPES, 1.0 *M* NaCl pH 6.1. The suspension is filtered through 0.45-μm filter, equilibrated to 20 m*M* PIPES, 1.0 *M* NaCl pH 6.1 on a PD-10 column and used as a starting sample for IMAC. Column: 6.5×1.0 cm. I.D. (5.1 ml) Fe(III)–Chelating Sepharose FF was equilibrated with 5 column volumes of 20 m*M* PIPES, 1.0 *M* NaCl pH 6.1. Chromatography: Flow-rate, 0.66 ml/min. 1: Loading of 7.9 ml of the sample and washing with 20 m*M* PIPES, 1.0 *M* NaCl pH 6.1. 2: Washing with 20 m*M* PIPES, 1.0 *M* NaCl pH 6.9. 3: Elution with 0.02 *M* sodium phosphate, 1.0 *M* NaCl pH 7.0. 4: Elution with 0.1 *M* EDTA pH 7.0.

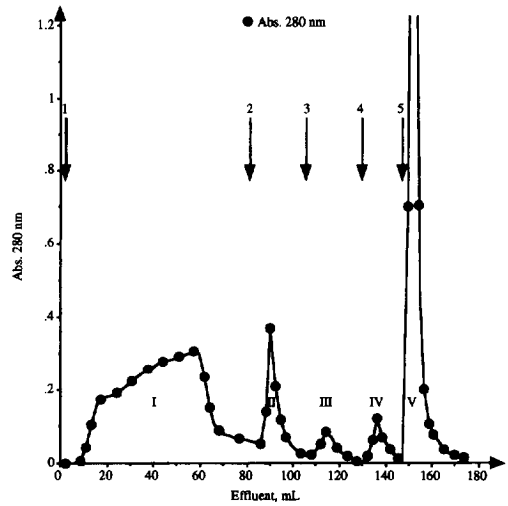

Fig. 6. Chromatogram of Peak II (material from the experiment referred to in Fig. 5) on Eu(III)–TED–agarose. Sample: 50 ml of peak II (Fig. 5) is saturated to 2.0 *M* NaCl, 0.2 *M* $CaCl_2$ by adding solid salts. Column: 6.3×0.5 cm I.D. (5 ml) TED–Novarose (High) was charged with Eu(III), washed with water and equilibrated with 5 column volumes of 0.1 *M* Tris–HCl, 2.0 *M* NaCl, 0.2 1 *M* $CaCl_2$ pH 7.5. Chromatography: Flow rate, 0.66 ml/min. 1: Loading of 50 ml of the sample and washing with 0.1 *M* Tris–HCl, 2.0 *M* NaCl, 0.2 *M* $CaCl_2$ pH 7.5. 2: Elution with 0.1 *M* Tris–HCl, 2.0 *M* NaCl, 0.6 *M* Na_2SO_4 pH 7.5. 3: Elution with 0.1 *M* Tris–HCl, 2.0 *M* NaCl, 0.6 *M* Na_2SO_4, 40 m*M* malonate pH 7.5. 4: Elution with 0.2 *M* potassium phosphate, 3.0 *M* NaCl, 0.2 *M* citrate pH 7.0. 5: Elution with 0.1 *M* EDTA pH 7.0.

column at pH 6.9 (peak II in Fig. 5); this indicates weak interaction with immobilized Fe(III). Many other proteins have similar affinity as can be seen in Fig. 7, lane 3. Although only 3.1-fold purification was achieved in this step, it removed a major portion of albumin and all of the phosphorylase [22], a prerequisite for the success of the next IMAC step on immobilized Eu(III).

Step 2: The material from peak II (Fig. 5) was saturated to 2.0 *M* NaCl, 0.2 *M* $CaCl_2$ by adding solid salts and was used as a starting material for IMAC on Eu(III)–TED–agarose. The loading and elution conditions were chosen after a series of experiments were conducted to determine the highest possible recovery and selectivity. The decrease of the initial NaCl concentration from 3.0 *M* to 2.0 *M* was made in order to achieve complete elution of CaM with 0.2 *M* potassium phosphate, 3.0 *M* NaCl, 0.2 *M* citrate pH 7.0. An additional preliminary step before the elution of the adsorbed CaM was introduced (Step 3, elution with 0.1 *M* Tris–HCl, 2.0 *M* NaCl, 0.6 *M* Na_2SO_4, 40 m*M* malonate pH 7.5) that resulted in desorption of proteins that were more weakly adsorbed to Eu(III)–TED–agarose than CaM (peak III, Fig. 6). The nature of these proteins is unknown, but one could suppose that they also have calcium-binding properties.

After optimization of the loading and elution conditions for the second step, the purification factor increased to 28 and resulted in electrophoretically pure CaM (peak IV in Fig. 6 and Fig. 7, lane 4). The purified CaM was analyzed with SDS–gel electrophoresis (Fig. 7) and IEF (Fig. 8). The p*I* was determined to be 3.8, in agreement with the reported value of 3.85 [25].

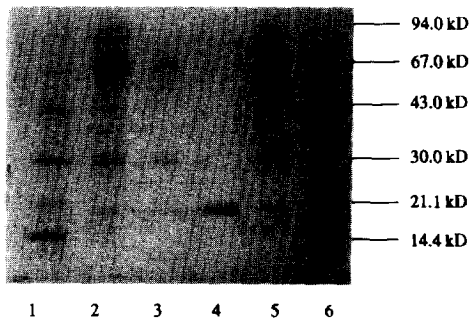

Fig. 7. Analyses of various fractions by SDS–gel electrophoresis. The lanes correspond to: 1=low-molecular-weight protein kit (Pharmacia); 2=original sample (crude CaM after IEC); 3=peak II, Fig. 5; 4=peak IV, Fig. 6; 5=original sample (crude CaM after IEC); 6=low-molecular-weight protein kit (Pharmacia).

4. Discussion

This paper describes the use of lanthanides as a substitute for calcium in purification of calcium-binding proteins with IMAC. This study was undertaken to determine if these metal ions have an affinity similar to that of Ca^{2+} towards Ca^{2+}-binding proteins in immobilized form as in solution and whether such an affinity could be exploited.

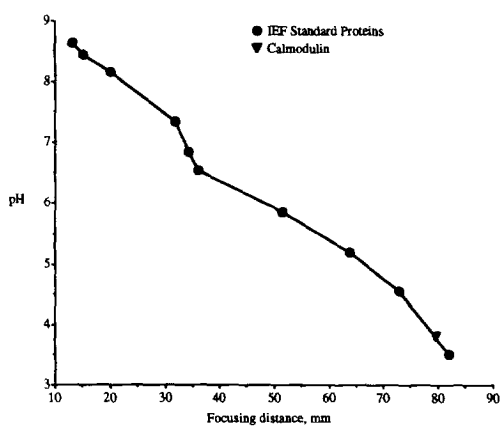

Fig. 8. Diagram obtained from isoelectric focusing of purified calmodulin. The points correspond to: (▲) calmodulin (peak IV, Fig. 6) after desalting. (●) IEF standard proteins from top to bottom are as follows: 1, lentil lectin basic, p*I* 8.65; 2, lentil lectin middle, p*I* 8.45; 3, lentil lectin acidic, p*I* 8.15; 4, myoglobin basic, p*I* 7.35; 5, myoglobin acidic, p*I* 6.85; 6, human carbonic anhydrase B, p*I* 6.55; 7, bovine carbonic anhydrase B, p*I* 5.85; 8, β-lactoglobulin, p*I* 5.2; 9, soybean trypsin inhibitor, p*I* 4.55; 10, amyloglucosidase, p*I* 3.5.

Although considerable knowledge has been accumulated on the properties of immobilized transition metal ions [11,26], very little is known about those of the group of so-called "hard" metal ions. From this group, only immobilized Fe(III) has been thoroughly investigated. The specificity of this metal ion is believed to be towards the oxygen of the carboxy- and phosphate groups (of the phosphorylated proteins) [27–29]. Recently it was proposed that clusters of histidines might contribute to binding to immobilized iron ions at pH values above neutral [30]. It is interesting to note that the presence of EF domains in the protein structure (where the binding of Ca^{2+} is attributed mainly to the carboxylic groups of aspartic and glutamic acid) does not result in a strong binding to immobilized Fe(III) as indicated by the complete elution of CaM at pH values less than 7.0.

In contrast to immobilized Fe(III), immobilized Ca(II) and lanthanides show higher capacity at pH values above 7.0 [31]. The capacity is dependent upon a high salt concentration. This is probably explained by a salting-out effect that such high concentrations have on the protein structure resulting in an energetically more efficient approach between the EF hands and the immobilized metal ions. The finding that immobilized lanthanides have a higher capacity at pH values above 7.0 could be explained to some extent by the results reported by Henzl et al. on the involvement of the deprotonated serine side chain in the interaction of parvalbumin with Eu(III) [18].

The effect of Ca(II) on the capacity of adsorbents based on immobilized lanthanides is interesting. A possible reason for this effect could be the "sandwich" model in which the immobilized lanthanide ion serves as an initial anchor with which the first Ca^{2+}-binding molecule interacts, followed by formation of bridges between CaM molecules of the types depicted in Fig. 9a and b. The first proposed type of regulation described in Fig. 9a is more likely with the Ca(II) ions affecting the folding of the protein and exposing binding sites with affinity for another

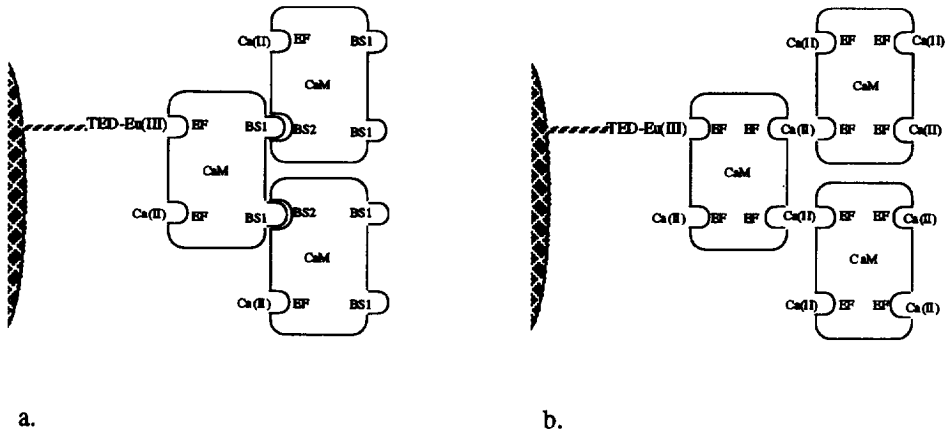

Fig. 9. Proposed mechanisms of the effect of Ca(II) ions on the capacity of adsorbents with immobilized lanthanides. The assignments are: EF, EF domain; BS1, binding site 1 (formed or exposed in presence of Ca(II)); BS2, binding site 2 (might or might not be effected by Ca(II)). (a) The binding of Ca(II) to the EF domains leads to exposure of BS1 with affinity for another region (BS2) of a second CaM molecule. (b) The bound Ca(II) is shared between two EF domains from different molecules.

region of the second molecule of CaM. The occurrence of the second type (Fig. 9b) has a higher statistical probability compared with protein–protein interaction in free solution, but no indication of similar behavior (aggregation of CaM molecules in presence of Ca(II) ions) has been reported to the knowledge of the authors of this manuscript.

CaM has been isolated by several procedures. Kakiuchi and coworkers used DEAE–Sepharose and fluphenazine–Sepharose [32] and Teo et al. used DEAE–cellulose and Sephadex G-100 [33]. A one-step chromatographic purification of CaM has also been described [21] that employs a rather harsh heat pretreatment and isoelectric precipitation preceding the chromatography.

Solving of any particular separation problem involves two major steps. Preliminary screening of the separation principles in order to determine those best suited and optimization of the conditions at which any particular principle is applied. The choice of the separation conditions is always a compromise between maximal capacity, yield and selectivity. In this respect, the purification procedure reported here exemplifies this statement. The maximum operational capacity of immobilized Eu(III) towards CaM can be achieved at 3.0 *M* NaCl (0.1 *M* Tris–HCl, 0.2 *M* $CaCl_2$ pH 7.5)-12.9 mg/ml gel. Instead we chose as a starting buffer 2.0 *M* NaCl (0.1 *M* Tris–HCl, 0.2 *M* $CaCl_2$ pH 7.5) with operational adsorption capacity of 4.8 mg/ml gel. This results in higher selectivity (the purification ratio increases from 5 to 28) and complete elution of CaM from the column in a single peak.

In conclusion, the results reported in this paper confirm the idea that lanthanides can be used as Ca(II) substitutes in IMAC. The capacity of such adsorbants is close to those reported for other immobilized metal ions. The experimental data reported here can be used for development of a general method for purification of Ca(II)-binding proteins that possess surface-exposed EF domains as well as a basis for further study on the effect of Ca(II) presence in the mobile phase.

5. Notation

CaM	Calmodulin
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
IMAC	Immobilized metal ion affinity chromatography
PIPES	Piperazine-N,N′-bis[2-ethanesulfonic acid]
TED	Tris(carboxymethyl)ethylenediamine
Tris	Tris(hydrohymethyl)aminomethane

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