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Calcium-modulated conformational affinity chromatography

Application to the purification of calmodulin and S100 proteins

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ABSTRACT

The purification of proteins by affinity chromatography is based on their highly specific interaction with an immobilized ligand followed by elution under conditions where their affinity towards the ligand is markedly reduced. Thus, a high-degree purification by a single chromatographic step is achieved. However, when several proteins in the crude mixture share affinity to a common immobilized ligand, they may not be resolved by affinity chromatography and subsequent "real" chromatographic purification steps may be required. It is shown that by using properly selected gradient elution conditions, the affinities of the various proteins towards the immobilized ligand may be gradually modulated and their separation may be achieved. This is exemplified by the isolation and separation of a group of Ca^{2+}-activated proteins, Calmodulin, S100a and S100b, from bovine brain extract, using a melittin–Eupergit C affinity column which is developed with Ca^{2+}–chelator gradients. As expected, separation of the three proteins into individual peaks, eluted in order of increasing affinity to the matrix, was obtained. Sigmoid selectivity curves calculated from the elution volumes under different elution conditions for each of the proteins were obtained, illustrating the chromatographic behaviour of the gradient affinity separation system.

INTRODUCTION

The high resolving power of affinity chromatography is derived from highly specific interactions between an immobilized ligand and a certain protein present in a crude mixture of foreign proteins which allow its selective isolation [1,2]. However, when the immobilized ligand recognizes more than a single protein in the crude preparation, their separation by affinity chromatography may not be feasible. In some instances, especially when the ligand is a small and simple molecule, this problem may be overcome by a gradual increase in the free ligand concentration in the elution buffer [3]. Such an approach may not be applicable when the ligand is an expensive peptide or a protein (*e.g.*, antibodies) or when no contamination of the purified protein preparation by the soluble ligand is allowed.

A well known case of such group-specific recognition by a common ligand is the Ca^{2+}-dependent interaction of melittin, a bee venom peptide, with a number of different Ca^{2+}-binding proteins. Calmodulin [4,5] and S100 proteins [6,7], present in bovine brain at high concentrations, possess high affinities towards melittin (10^7–10^9 l/mol [8]) in the presence of Ca^{2+} ions. Removal of the Ca^{2+} ions causes marked conformational changes in these proteins, which strongly reduce their affinity towards the ligand [9,10]. Columns of immobilized melittin have been used for the isolation of fractions containing

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Ca^{2+}-binding proteins from tissue extracts by affinity chromatography [11,12]. However, these proteins were not separable from each other by affinity purification and successive chromatographic steps were necessary (*e.g.*, chromatography on a mercury–agarose column [13]).

We propose that in such instances, a gradual change of the elution conditions should lead to a concomitant decrease in the affinities of the various proteins to the immobilized ligand and their subsequent release from the matrix. In this paper the usefulness of this approach is illustrated by its application to the separation of calmodulin, S100a and S100b, using Ca^{2+}–chelate gradients. Since the three proteins differ in their binding constants to Ca^{2+} ions [4,14,15], their release from a column of Eupergit C-immobilized melittin is expected to occur at different Ca^{2+} concentrations at which the conformational change of each protein takes place.

EXPERIMENTAL

Materials

Melittin, calmodulin, S100a, S100b, ethylene glycol bis(β-aminoethyl) tetraacetic acid (EGTA) and sodium citrate were purchased from Sigma (St. Louis, MO, USA), dansyl chloride (sequencing grade) from Pierce (Rockford, IL, USA) and Eupergit C from Rohm (Darmstadt, Germany).

Labelling of calmodulin

Calmodulin was iodinated with I^{125} (23.4 μCi/μg) by Rotem (Beer Sheva, Israel). Dansylation of calmodulin was carried out as described [16].

Preparation of brain extract

Bovine brain (300 g, fresh or after storage at −70°C) was homogenized in a Waring blender for 3 min in 600 ml of cold extraction buffer (0.1 *M* Tris–HCl buffer, pH 7.5, containing 2 m*M* EGTA). The homogenate was centrifuged at 20 000 *g* for 30 min at 4°C in an RC-5C Sorvall centrifuge (Dupont, Wilmington, DE, USA). The supernatant was collected and recentrifuged twice under the same conditions. The final supernatant was dialysed in a Spectrum dialysis bag (molecular mass 3000 cut-off) against the extraction buffer. The extract was kept at −20°C.

Preparation of melittin–Eupergit C conjugate

Prior to its immobilization on Eupergit C, melittin was purified from amine-containing salts by gel filtration on a Bio-Gel P-4 column (Bio-Rad Labs., Richmond, CA, USA) developed in phosphate-buffered saline (PBS) (pH 7.4). The melittin-containing fractions were collected and the peptide (5 mg) was immobilized by reaction of its amino groups with oxirane moieties of Eupergit C30N beads (2 g) as decribed previously [17]. For determination of the amount of melittin bound to the matrix, a sample of the conjugate (50 mg) was extensively washed with doubly distilled water and then subjected to total acid hydrolysis by 6 *M* HCl at 121°C for 22 h. After centrifugation the supernatant was neutralized and subjected to amino acid analysis (hydrolysis and analysis were performed by AminoLab, Rehovot, Israel).

Determination of the association constant of immobilized melittin with calmodulin

Fractions (10 μl) of iodinated calmodulin ($5 \cdot 10^{-8}$ *M*, 9000 cpm) were mixed with increasing amounts (0.3–3 mg) of melittin–Eupergit C conjugate ($3.2 \cdot 10^{-6}$–$3.2 \cdot 10^{-5}$ *M* final concentration) and diluted to 50 μl with Tris–HCl buffer (pH 7.5) containing 5 m*M* $CaCl_2$. Alternatively, the concentration of melittin–Eupergit C conjugate was kept constant at $3.2 \cdot 10^{-5}$ *M* and the concentration of calmodulin was varied between $5 \cdot 10^{-10}$ and $1 \cdot 10^{-7}$ *M* (final concentration), each fraction containing 9000 cpm of radiolabelled calmodulin. As the iodinated calmodulin, in contrast to the native protein, tended to adsorb on the surface of the melittin–Eupergit C beads, they were treated with 1% ovalbumin in PBS for 24 h at room temperature, prior to the experiment to avoid non-specific adsorption. The labelled calmodulin was incubated with the beads for 30 min at room temperature in Tris–HCl buffer (pH 7.5) containing 5 m*M* $CaCl_2$. The beads were then washed with the same buffer and the amount of bound protein was determined by measuring the bead-associated radioactivity at zero time and at the end of the reaction.

In a parallel experiment, the binding of dansylated calmodulin ($1 \cdot 10^{-7}$–$1 \cdot 10^{-5}$ *M* final concentration) was analysed under similar experimental conditions except that the melittin–Eupergit C beads were not pretreated with ovalbumin. In this experi-

ment, dansylated calmodulin samples were incubated at room temperature with 24-mg fractions of melittin–Eupergit C for 30 min in 400 μl (final volume) of Tris–HCl buffer (pH 7.5) containing 5 m*M* $CaCl_2$. The fluorescence of the reaction mixture supernatant was measured at zero time and at the end of the incubation period using a Perkin-Elmer (Beaconsfield, UK) Model LC-50 spectrofluorimeter ($\lambda_{ex} = 340$, $\lambda_{em} = 460$ nm). The binding of calmodulin to immobilized melittin in the absence of Ca^{2+} ions was carried out under the same conditions except that EGTA (2 m*M*) replaced the $CaCl_2$ in the reaction mixture.

High-performance affinity chromatography

Eupergit C-conjugated melittin was packed into a stainless-steel high-performance liquid chromatographic (HPLC) column (6 × 0.4 cm I.D.) (Knawer, Bad Homburg, Germany). The column was connected to a Gilson (Villiers le Bel, France) HPLC system equipped with an Pharmacia–LKB (Uppsala, Sweden) Model 2140 diode-array detector. Standards or brain extract samples were applied to the column in 0.1 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $CaCl_2$ at a flow-rate of 0.2 ml/min. Prior to application of tissue extract samples to the column they were centrifuged for 10 min at 12 000 *g* and the supernatant was diluted 1:1 with the loading buffer. Elution of the Ca^{2+}-dependent proteins with EGTA [2 m*M* in Tris–HCl buffer (pH 7.5)], citrate (5 m*M* in the same buffer) or a gradual mixture of them was carried out at 0.5 ml/min.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE in the presence of β-mercaptoethanol was performed as described by Laemmli [18].

Determination of chromatographic parameters

In order to determine the capacity factors (k') and construct selectivity (α) curves for calmodulin and the S100 proteins on the melittin–Eupergit C column, samples of each of these proteins (20 μg in 50 m*M* Tris–HCl buffer (pH 7.4) containing 5 m*M* $CaCl_2$) were loaded on a column equilibrated with the same buffer at a flow-rate of 0.5 ml/min. After 10 min the column was washed with elution buffers, composed of a mixture of citrate [5 m*M* in 0.1 *M* Tris–HCl buffer (pH 7.5)] and EGTA (2 m*M* in the same buffer) at various ratios (0–100% EGTA). The elution volume (V_e) of each protein by the various elution buffers were recorded and compared with the void volume of the column (V_0), determined as the elution volume of an unretarded protein (IgG). After each run the column was washed with 2 m*M* EGTA in the above buffer and then re-equilibrated with the Ca^{2+}-containing buffer.

The capacity and selectivity factors were calculated as $k' = V_e/V_0 - 1$ and $\alpha = V_0/V_e$, respectively.

RESULTS

Preparation of melittin–Eupergit C affinity column

Coupling of melittin to Eupergit C beads by reaction of its amino groups with oxirane groups of the matrix yielded an active immobilized melittin preparation. When 2.5 mg of melittin were allowed to interact with 1 g of Eupergit C beads for 16 h at 4°C, 80% of the peptide was covalently conjugated to the matrix as determined by amino acid analysis of the matrix after total acid hydrolysis. The calmodulin-binding capacity of the conjugate was determined as the amount of calmodulin bound to the matrix in presence of Ca^{2+} ions (5 m*M*) and then eluted with 2 m*M* EGTA. It was found that 200 μg of immobilized peptide were capable of binding 250 μg of calmodulin, indicating that the matrix-conjugated melittin retained about 20% of its maximum theoretical activity (assuming equimolar binding of the peptide to calmodulin). The decrease in the apparent binding activity of the immobilized melittin compared with the peptide in solution was attributed to binding of part of its molecules within areas of the porous matrix which are inaccessible to calmodulin.

Determination of the apparent binding constant of calmodulin to the immobilized melittin

The affinity of calmodulin to the immobilized melittin was determined by a Scatchard analysis of the binding of radio- or fluorescent-labelled calmodulin to the matrix in presence of Ca^{2+} ions. The experiment was carried out by determining the degree of binding of a constant amount of calmodulin to increasing amounts of Eupergit C-conjugated melittin or increasing concentrations of labelled calmodulin to a constant amount of immobilized melittin. Linear Scatchard plots were obtained for both labelled

proteins, yielding binding constants of $4.6 \cdot 10^7$ and $0.75 \cdot 10^7$ l/mol for the iodinated and dansylated calmodulin preparations, respectively (Fig. 1). These values are about one order of magnitude lower than published values for the interaction of calmodulin with soluble melittin [8].

The molar fraction of active melittin on the matrix could be determined by the above analysis only for the dansylated protein and was found to be 0.17. The corresponding value obtained by the radiolabelled protein was about 500 times lower. This effect was attributed to the pretreatment of this preparation with ovalbumin to reduce non-specific interactions of the iodinated calmodulin with the matrix, apparently causing masking of most of the conjugated melittin molecules.

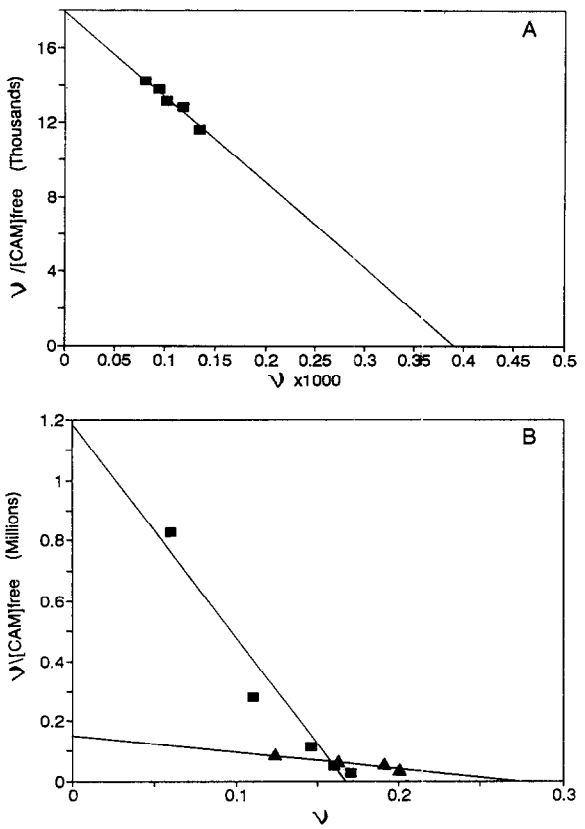

Fig. 1. Scatchard plots for the binding of calmodulin to Eupergit C-conjugated melittin. (A) Binding of iodinated calmodulin to the immobilized ligand was determined by measuring the matrix-associated radioactivity at zero time and after incubation for 30 min in PBS (pH 7.4). (B) Binding of dansylated calmodulin in (■) the presence or (▲) the absence of Ca^{2+} ions was followed by measurement of the fluorescence of the supernatants before and after the reaction (see Experimental for details). The experimental binding parameter ν is defined as the molar ratio between the bound calmodulin and the total melittin concentrations.

Scatchard analysis performed for the binding of dansylated calmodulin to immobilized melittin in the presence of EGTA (Fig. 1B) yielded a binding constant of $5 \cdot 10^5$ l/mol, indicating that the immobilized melittin is capable of calmodulin binding as reported in the literature for soluble melittin. The higher n value obtained in this case (0.27) may indicate a higher stoichiometry than 1:1 for the melittin:calmodulin complex as cited in the literature [10].

HPLC affinity purification of Ca^{2+}-binding proteins

Purification of calmodulin and other Ca^{2+}-dependent proteins by HPLC affinity chromatography on Eupergit C-immobilized melittin was first attempted using a commonly reported procedure: loading of the proteins on the column in presence of a high concentration (5 m*M*) of Ca^{2+} ions and elution of the specifically bound proteins with EGTA (2 m*M*), which is a strong chelator for Ca^{2+} ($K_a = 10^{11}$ l/mol [19]). Commercial pure calmodulin loaded on the column in the presence of Ca^{2+} ions was indeed recovered by washing with EGTA as a sharp peak (Fig. 2A). When a sample of bovine brain extract was applied to the column and run under the same experimental conditions, a similar peak was obtained (Fig. 2B). This peak, however, contained a mixture of Ca^{2+}-binding proteins, especially calmodulin and S100 proteins as indicated by SDS-PAGE (Fig. 3) and confirmed by affinity chromatography of this fraction on an agarose–mercury column (data not shown).

As affinity chromatography using EGTA as the eluent failed to separate calmodulin and the S100 proteins, we attempted their separation by application of Ca^{2+}–chelator gradients. The different Ca^{2+}-dependent proteins were expected to elute from the column according to their respective Ca^{2+}-binding constants (Table I). Application of a linear gradient between Ca^{2+} (5 m*M*) and EGTA (2 m*M*) over 20 min resulted in the elution of a poorly resolved double-headed peak containing the three proteins (Fig. 2C). When EGTA was replaced with sodium citrate, ($pK_{a_{Ca}} = 3.5$ [19]), no protein was eluted from the column even on prolonged washing

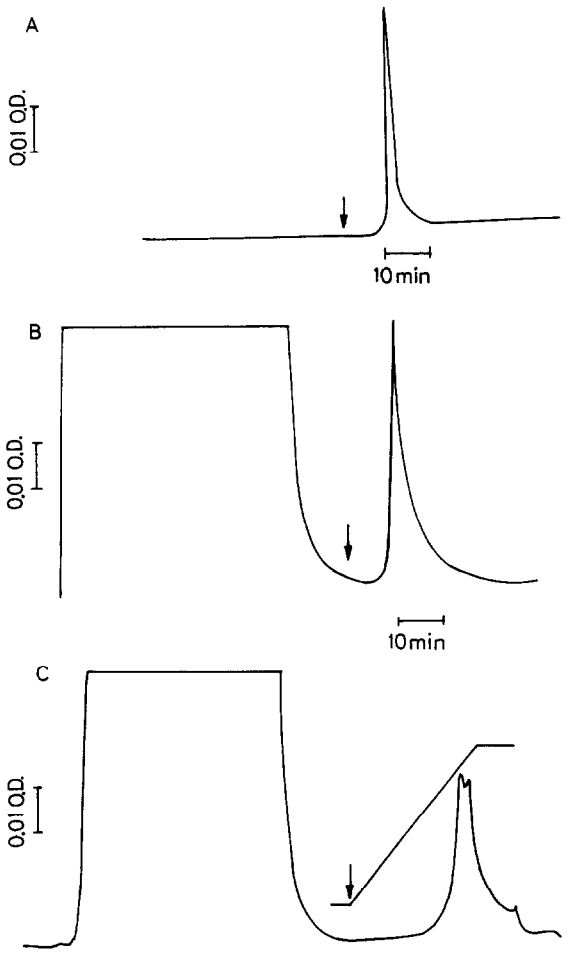

Fig. 2. Affinity chromatogram of (A) pure calmodulin and (B, C) bovine brain extract on a column of melittin–Eupergit C using step (A, B) or gradient (C) elution. Samples were applied to the column in 0.1 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $CaCl_2$. The arrows mark the application of 2 m*M* EGTA in the same buffer (A, B) or a linear gradient over 20 min of 5 m*M* $CaCl_2$ (buffer A) and 2 m*M* EGTA (buffer B). Loading of the samples was at 0.2 ml/min and elution was at 0.5 ml/min.

(data not shown). Unexpectedly, subsequent washing of the column with EGTA failed to release the bound proteins. These were released only when the column re-washed with the Ca^{2+}-containing buffer and then with EGTA. This effect was attributed to interaction of the proteins with the ligand even in the absence of Ca^{2+} ions, which is apparently slower. Thus, prolonged incubation of the protein–melittin matrix with sodium citrate may have resulted in rearrangement of the interaction of the three proteins with the immobilized melittin rendering the complexes insensitive to EGTA. Interaction between calmodulin and melittin in the absence of Ca^{2+} ions has been described previously [10].

In order to achieve a higher chelating power, elution of the Ca^{2+}-dependent proteins from the melittin–Eupergit C column was attempted by application of citrate and EGTA mixtures. In this set of experiments, each of the three proteins, or a sample of brain extract, were applied to the column in presence of 5 m*M* Ca^{2+} ions, and after a brief wash with citrate buffer a gradient between citrate and EGTA was applied (Fig. 4). Application of calmodulin and each of the two S100 proteins separately to the column and elution with the citrate–EGTA gradient led to their separation in accordance with their relative affinities to Ca^{2+} (Table I). Consecutive loading of a sample of bovine brain extract (1 ml) on the column and elution under the same conditions resulted in well separated and sharp peaks of the three proteins (Figs. 4B and 3).

Chromatographic properties of the affinity separation system

In order to illustrate the chromatographic characteristics of the gradual affinity separation system, the capacity factors of each of the proteins were determined and selectivity curves were constructed. As shown in Fig. 5, sigmoid selectivity curves with mid-points at about 50% EGTA were obtained for the two S100 proteins. In contrast, a sharp selectivity curve was obtained for calmodulin at 70–80% EGTA. These values roughly correspond to the values at which elution of the three proteins was observed under gradient conditions (Fig. 4). It is pertinent to note that despite the close similarity of the selectivity curves of the two S100 proteins, their

TABLE I

ASSOCIATION CONSTANTS OF Ca^{2+}-DEPENDENT PROTEINS WITH Ca^{2+} IONS CITED IN THE LITERATURE

Protein	K_a (l/mol)	Ref.
S100a	$4 \cdot 10^4$	14
S100b	$1 \cdot 10^4$	14
Calmodulin	$1 \cdot 10^7$	4

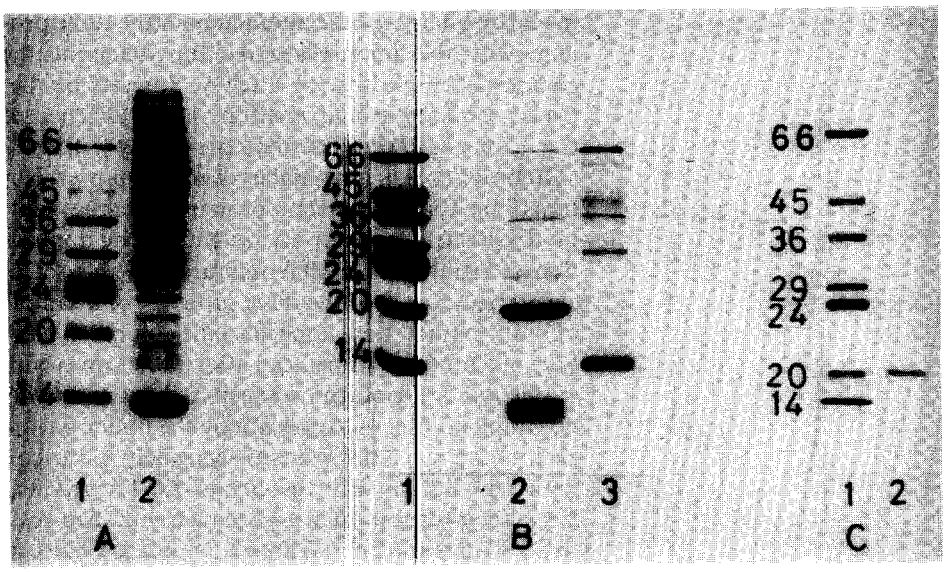

Fig. 3. Polyacrylamide gel electrophoresis. A1, B1 and C1 are protein markers. A2 is a sample of the crude brain extract. B2 and B3 are samples of the proteins eluted with EGTA and the flow-through of the column during loading, respectively. C2 is a sample of the calmodulin peak obtained by citrate–EGTA gradient elution (see Fig. 4).

baseline separation was achieved under the gradient elution conditions.

The melittin–Eupergit C column could be used repetitively for the purification of calmodulin and S100 proteins for 2–3 months (50–100 runs) before fouling of the column and loss of activity were observed. The activity of a fouled column could be fully recovered by washing it with 8 *M* urea in 0.1 *M* Tris–HCl buffer (pH 7.5) for 20 min, followed by a

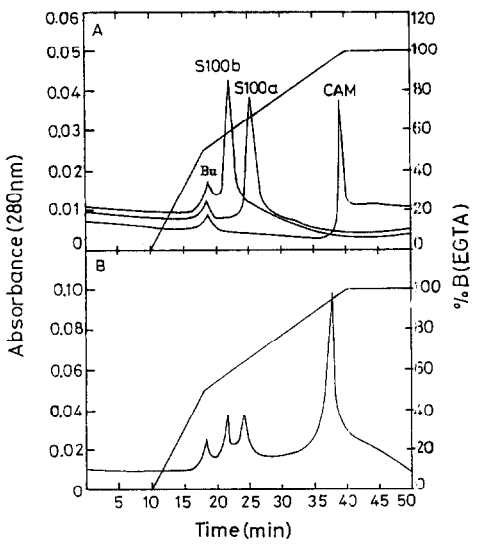

Fig. 4. Elution profiles of (A) standards and (B) tissue extract Ca^{2+}-dependent proteins from the melittin–Eupergit C column using a gradient between 0.05 *M* sodium citrate (buffer A) and 0.02 *M* EGTA (buffer B) in 0.1 *M* Tris–HCl buffer (pH 7.5) as indicated. The chromatogram of the loading step is not shown.

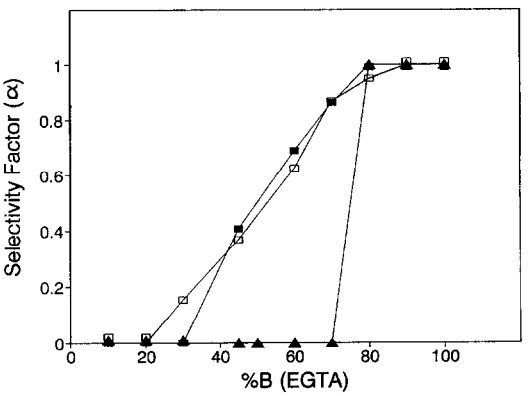

Fig. 5. Selectivity curves for (▲) calmodulin, (□) S100a and (■) S100b on the melittin–Eupergit C column. Samples of each protein (100 μg) were loaded on the column in 0.1 *M* Tris–HCl buffer (pH 7.5) containing 2 m*M* $CaCl_2$ and eluted with a mixture of sodium citrate (5 m*M*) and EGTA (2 m*M*) at the indicated ratio. Elution times were recorded and the capacity and selectivity factors were calculated accordingly.

30-min wash with 0.1 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $CaCl_2$.

DISCUSSION

During the last decade, affinity chromatography has become a major tool in protein purification. The high specificity of the immobilized ligand towards a certain protein in a crude mixture allows the rapid isolation of that protein, achieving high purification factors in a single separation step. However, affinity chromatography, despite its name, is not a "real" chromatographic process as the various proteins in the sample are separated on an "all or none" basis according to their recognition of the ligand, and not on the basis of selective partitioning between the stationary and mobile phases. Hence a mixture of proteins which interact with a common ligand may not be separated by classical affinity chromatography using the same immobilized ligand.

A well known case is the interaction of Ca^{2+}-dependent proteins with target proteins and peptides. Several such proteins often recognize a common ligand, the affinities modulated by conformational changes induced in their molecules by the presence or absence of Ca^{2+} ions. We felt that this system might serve as an excellent model for the development of an affinity chromatographic method which would separate such proteins according to their increasing affinity towards the immobilized ligand.

Previously, it has been shown that monoclonal antibodies which specifically recognize Ca^{2+}-induced conformational changes in calmodulin could be used for the separation of two conformers by immunoaffinity chromatography [20,21]. Such an approach, which is also applicable to other proteins [22], is very efficient but may be adopted only when such antibodies are available. Usually, the preparation of conformation-specific antibodies is complicated by the close structural similarity between the different conformers and by the difficulties in obtaining a certain conformer in a pure state.

In this work we have demonstrated the purification of Ca^{2+}-activated proteins from bovine brain extract using a melittin–Eupergit C column. Scatchard analysis of the binding of calmodulin to the immobilized melittin showed that (a) its binding constant to calmodulin in presence of Ca^{2+} ions was about one order of magnitude lower than the values cited in the literature for melittin in solution and (b) about 20% of the immobilized ligand retained its activity when immobilized. Some binding of calmodulin to the immobilized melittin was also detected in the absence of Ca^{2+} ions and the binding constant calculated was similar to that cited in the literature [10].

Gradient elution conditions using citrate–EGTA mixtures were developed in order to achieve a baseline separation between the three proteins tested, calmodulin, S100a and S100b, purified from bovine brain extract. Apparently, the separation of these proteins on the melittin–Eupergit C column seems to be governed by two opposing rules: (a) Ca^{2+} ions have to be removed gradually to allow a gradual change in the affinities of the different proteins to the matrix, and (b) removal of Ca^{2+} ions should be fast enough to minimize rearrangement of the protein–melittin complexes into an EGTA-insensitive form. Under the developed conditions, the three proteins tested were well resolved, eluting in the order expected from their affinities to Ca^{2+}.

A well known characteristic of a chromatographic system is the separation of the solutes according to typical selectivity curves which determine the distribution of each solute between the stationary and mobile phases. The difference between the selectivity (α) curves (usually of sigmoid shape) of the various solutes allow their chromatographic separation. In affinity chromatography, in contrast, separation is based on loading under conditions where $\alpha = 0$ for the protein of interest only and elution under conditions where $\alpha = 1$. The chromatographic behaviour of the gradient-elution affinity separation system was illustrated by the determination of the capacity and selectivity factors for each of the tested proteins under different elution conditions. At least for the two S100 proteins a gradual increase in the column selectivity with increasing EGTA concentration was observed, indicating a true chromatographic exchange between the stationary and mobile phases of the column. Calmodulin, in contrast, showed a sharp increase in the selectivity factor from $\alpha = 0$ to $\alpha = 1$, which suggested a non-chromatographic behaviour of this protein on the melittin–Eupergit C column. Unexpectedly, the selectivity patterns of the two S100 proteins were very similar to each oth-

er. Nevertheless, the small differences allowed a baseline separation between the two proteins by gradient elution.

Hence the possibility of turning affinity separation into a "real" chromatographic process, involving the separation of several proteins which recognize the common immobilized ligand, has been illustrated. This principle may also be applied to other types of affinity chromatography, *e.g.*, the separation of isoenzymes or different conformers by immunoaffinity chromatography.

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