Enhancement of Calcium Sensitivity of Lipocortin I in Phospholipid Binding Induced by Limited Proteolysis and Phosphorylation at the Amino Terminus as Analyzed by Phospholipid Affinity Column Chromatography*

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A phospholipid column was prepared by coating siliconized porous glass beads with phospholipids. The analysis of the Ca** requirement of lipocortin I and its derivatives in the binding to phospholipids was carried out with this column. The Ca** concentration required for 50% binding to the phospholipid column at room temperature was about 30 µM for lipocortin I, while that was reduced to 15 µM when lipocortin I was phosphorylated by the epidermal growth factor receptor/kinase, and a further reduction in the Ca** requirement was observed with proteolytic cleavage at the N-terminal region. Cathepsin D and calpain I (low calcium-requiring form of calcium-activated neutral protease) rapidly cleaved human placental lipocortin I at Trp-12 and Lys-26, respectively. These N-terminal-truncated proteins required only 5 µM Ca** for 50% binding to the phospholipid column. This enhancement of Ca** sensitivity by limited proteolysis was also observed for porcine lung lipocortin I. Essentially the same results were obtained when the Ca** sensitivities of the modified lipocortins I were analyzed using dispersed phospholipid vesicles instead of the phospholipid affinity column. Equilibrium dialysis indicated that the release of the N-terminal region markedly increased the affinity of lipocortin I for Ca** in the presence of phosphodiesterase, without any appreciable change of the number of Ca**-binding sites. Limited proteolysis by endogenous proteases such as calpain may be an important regulatory mechanism for the Ca** sensitivity of lipocortin I in phospholipid binding.

In addition to lipocortin I, several cytosolic Ca**/phospholipid-binding proteins such as lipocortin II (calpactin I heavy chain), endonexins, proteins I-III, calelectrins, chromobins, and placental anticoagulant proteins have been identified in many organs (6-11). Recent studies on their primary structures have shown that each Ca**/phospholipid-binding protein has a specific small N-terminal domain connected to a C-terminal core domain. The C-terminal core domain is highly homologous among these proteins and composed of four copies of a 70-amino acid repeating unit. These repeating units are thought to contain Ca**/phospholipid-binding sites (12-18).

No similarity has been found in the amino acid sequence of the N-terminal domain of these proteins. The N-terminal domain may confer a biological activity specific to each protein. In lipocortins I and II, this domain consists of 30 amino acids and has interesting biochemical properties. The N-terminal tail of lipocortin II contains a binding site for a 10-kDa protein (p10, calpactin I light chain) and phosphorylation sites by pp60** and protein kinase C (Ca**/phospholipid-dependent enzyme) (7, 19, 20). Proteolysis and phosphorylation at the tail are reported to cause a loss in the ability to associate with p10 and a decrease in the Ca** sensitivity of lipocortin II for phospholipid binding (21-23).

The N-terminal region of lipocortin I contains phosphorylation sites for the EGF receptor/kinase and protein kinase C, and is extremely susceptible to proteolysis similar to lipocortin II (5, 24-26). In contrast with lipocortin I, phosphorylation of lipocortin I by the EGF receptor/kinase is reported to enhance the Ca** sensitivity for phospholipid binding (27). In this study, we tested whether the proteolytic modification at the N-terminal domain also affects the Ca** sensitivity of lipocortin I. For the modifying protease, we have tested cathepsin D and calpain I. Cathepsin D was chosen, because this enzyme is present in a wide range of cells and may have a chance to act on lipocortins. Acidic conditions during the course of purification was noticed to accelerate the degradation of lipocortins. Calpain (EC 3.4.22.17) is a family of Ca**-dependent cysteine proteinases and is ubiquitously distributed.

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1 The abbreviations used are: EGF, epidermal growth factor; CPG, controlled pore glass; PCl, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; EGTA, ethylenebis(oxyethylene)-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PTH, phenylthiodydantoin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

2 Y. Ando, unpublished data.
in cytosolic fractions of various cells (for reviews, Refs. 28-30). Since calpain is known to associate with plasma membrane in a Ca\(^{2+}\)-dependent manner (31-33), the interaction between lipocortin and calpain may occur on the cytoplasmic surface of the plasma membrane in vivo. In order to analyze the Ca\(^{2+}\) requirement for the phospholipid binding of lipocortins, we performed affinity chromatography using a newly developed phospholipid-CPG column employing a decreasing Ca\(^{2+}\) gradient system.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Degradation of Lipocortin I by Cathepsin D and Calpain I**—Fig. 4 shows the results when human placental lipocortin I was subjected to digestion with cathepsin D at 30 °C and pH 4.5. PAGE analysis of the reaction mixture followed by Coomassie Blue staining showed that mild proteolysis converted 37-kDa lipocortin I (lane 1) to a 35.5-kDa form (lanes 2-6). The 35.5-kDa peptide was relatively insensitive to cathepsin D, but was further digested to a 34-kDa form by increasing the amount of cathepsin D and/or by the prolongation of incubation time (lanes 7 and 8). This proteolysis was inhibited by pepstatin A (2.5 μg) or by elevating the pH of the reaction mixture to pH 8.

When lipocortin I was incubated with calpain I at molar ratios of 80:1 (Fig. 5A) and 20:1 (Fig. 5B) in the presence of 0.2 mM Ca\(^{2+}\) at 30 °C for the indicated periods, time-dependent conversion of lipocortin I (37 kDa) to a 34-kDa form was observed. A typical limited proteolysis was observed; i.e. we could not observe any intermediate degradation products, which would migrate between 37 and 34 kDa, even in a milder proteolysis with a small amount of calpain and for a short incubation time (Fig. 5A), nor further digestion of the 34-kDa product occurred by increasing the amount of calpain and by the prolongation of the incubation time (Fig. 5B). The degradation of lipocortin I was completely prevented by well known calpain inhibitors such as EDTA (1 mM), calpastatin (3 units), or leupeptin (5 μg).

**Comparison of Ca\(^{2+}\) Requirements for Phospholipid Binding of Intact and N-Terminal-truncated Lipocortins I**—Human placental lipocortin I and des 1-26 lipocortin I were applied to the affinity column in the presence of 100 μM Ca\(^{2+}\), and eluted with linear gradients of decreasing CaCl\(_2\) and correspondingly increasing EGTA at room temperature (Fig. 7). Intact lipocortin I was eluted at 32 μM Ca\(^{2+}\), while the elution...
of des 1–26 lipocortin I was markedly retarded and was observed at 4 μM Ca\(^{2+}\). When the mixture of des 1–12 lipocortin I and des 1–26 lipocortin I was applied to the column, they were eluted in a single protein peak, but SDS-PAGE analysis showed that des 1–12 lipocortin I was eluted slightly earlier than des 1–26 lipocortin I (data not shown).

To see whether limited proteolysis reduces the Ca\(^{2+}\) requirement for the binding to phospholipid vesicles as well as that for the binding to the affinity column, we studied the Ca\(^{2+}\) sensitivity in the association of lipocortin I with dispersed PS vesicles (Fig. 9). Human placental lipocortin I or des 1–26 lipocortin I was incubated with PS vesicles at varying free calcium concentrations ranging from 0 to 100 μM. As expected from the experiments using the phospholipid affinity column (Fig. 7), about 80% of des 1–26 lipocortin I was found to be associated with the liposomes at 10 μM Ca\(^{2+}\), and the half-maximal association was observed at about 5 μM (Fig. 9). This was appreciably less than the Ca\(^{2+}\) concentration required for intact lipocortin I, which was 28 μM (Fig. 9).

**Equilibrium Dialysis**—In order to examine whether the enhancement of Ca\(^{2+}\) sensitivity of des 1–26 lipocortin I in phospholipid binding had an effect on Ca\(^{2+}\) affinity or stoichiometry, equilibrium dialysis of human placental lipocortin I and des 1–26 lipocortin I against \(^{45}\)Ca\(^{2+}\) was performed in the presence or absence of PS vesicles (Fig. 10). High binding of calcium to des 1–26 lipocortin I was observed even at relatively low concentrations of Ca\(^{2+}\) in the presence of PS, but not when PS was absent (Fig. 10A). Scatchard plots of lipocortin I and des 1–26 lipocortin I in the presence of PS extrapolated to 4.1 and 3.6 Ca\(^{2+}\)-binding sites per molecule, respectively, and the slopes of the lines indicated apparent Kd...
Porcine lung lipocortin I was phosphorylated using [γ-32P]ATP and a phospholipid-CPG column to/kinase, as described under "Experimental Procedures." Lipocortin I (37 kDa, 150 μg), 32P-labeled lipocortin I (37 kDa, trace amount), and des 1-26 lipocortin I (34 kDa, 150 μg) were applied to a phospholipid-CPG column (0.7 × 4.5 cm) and eluted with a decreasing Ca2+ gradient at 4 °C (A). The fractions were subjected to SDS-PAGE. Electrophoresed proteins were stained with Coomassie Blue (B) and autoradiographed (C). Samples are: lanes 1, fraction 16; lanes 2, fraction 18; lanes 3, fraction 20; lanes 4, fraction 22; lanes 5, fraction 24; lanes 6, fraction 26; lanes 7, fraction 28; lanes 8, fraction 30.

**TABLE I**

| Lipocortin | At 20-25 °C | At 4 °C |
|-----------|-------------|--------|
| Human placental | | |
| Lipocortin I | 31 ± 4.2 (6) | NT* |
| Des 1-12 lipocortin I | 6.3 (2) | NT |
| Des 1-26 lipocortin I | 4.2 ± 1.4 (5) | NT |
| Lipocortin II | 4.5 ± 2.2 (3) | NT |
| Porcine lung | | |
| Lipocortin I | 29 ± 4.4 (6) | 25 ± 3.2 (3) |
| Des 1-12 lipocortin I | 5.8 ± 2.2 (4) | NT |
| Des 1-26 lipocortin I | 4.9 ± 2.1 (5) | 4.3 ± 1.2 (3) |
| Phosphorylated lipocortin I | 15 (2) | 12 (2) |

*Not tested.

values of 90 and 18 μM, respectively (Fig. 10B).

Phospholipid-CPG Affinity Chromatography of Intact, Protease-treated, and Phosphorylated Lipocortin I Prepared from Porcine Lung—In order to test whether the enhancement of Ca2+ sensitivity is observed only with human lipocortin I or also with lipocortin I of other animal species, we purified porcine lung lipocortin I and investigated the effect of modifications of the N terminus on the Ca2+ sensitivity.

Phosphorylated porcine lung lipocortin I was prepared using the EGF receptor/kinase isolated from A431 cells. About 1% of native 37-kDa lipocortin I was phosphorylated, but none of des 1-26 lipocortin I was phosphorylated under our experimental conditions. This observation is consistent with previous reports that Tyr-21 is phosphorylated by the EGF receptor/kinase (5, 44). Porcine lung lipocortin I (37 kDa), 32P-labeled lipocortin I (37 kDa), and des 1-26 lipocortin I (34 kDa) were applied to the phospholipid-CPG column and eluted with a decreasing Ca2+ gradient at 4 °C or at room temperature. Fig. 11 shows one of these experiments. Phosphorylated lipocortin I was eluted between intact and des 1-26 lipocortins I as shown by Cerenkov counting (Fig. 11A) and autoradiography (Fig. 11C). While phosphorylation of lipocortin I decreased to half the amount of Ca2+ required for 50% association of the protein with the phospholipid column, much greater reduction of the Ca2+ requirement was observed for des 1-26 lipocortin I.

The Ca2+ concentrations required for 50% binding of lipocortin I and its derivatives to the phospholipid-CPG column are summarized in Table I. The order of Ca2+ sensitivity was des 1-26 lipocortin I > des 1-12 lipocortin I > phosphorylated lipocortin I > intact (native) lipocortin I, and this order was the same in experiments performed at both 4 °C and room temperature.

**DISCUSSION**

Lipocortins have been purified from various cells and organs such as A431 cells, intestinal mucosa, lung, and placenta (5, 20, 24, 44). Since the N-terminal regions of lipocortins are extremely sensitive to proteolytic cleavage, a rapid purification with fewer steps is highly recommended. In this paper, we have developed a phospholipid-CPG column and utilized it for affinity chromatography of lipocortins, instead of using repeated precipitation and extraction of lipocortins from the cellular particulate fraction, which is the standard method for the purification of lipocortins (5, 20, 24, 44). The intact...
The affinity of lipocortin I was rapidly purified from human placenta and porcine lung with phospholipid-EGTA column chromatography with a good yield.

For the analysis of Ca\textsuperscript{2+} dependence for lipocortins, phospholipid-EGTA column chromatography with CaCl\textsubscript{2}/EGTA gradients was found to be very useful. Human placental lipocortin I and des 1-26 lipocortin I required 31 \(\mu\)M and 4.2 \(\mu\)M Ca\textsuperscript{2+}, respectively, for 50% binding to the affinity column (Table I), and these values were comparable to those required for half-maximal association with PS vesicles: about 28 \(\mu\)M Ca\textsuperscript{2+} for intact lipocortin I and 5 \(\mu\)M Ca\textsuperscript{2+} for des 1-26 lipocortin I (Fig. 9). Lipocortin I required lower concentration of Ca\textsuperscript{2+} for phospholipid binding than lipocortin I in our experiments using the affinity column (Fig. 2), and such results have been reported using PS vesicles (7, 27). An affinity column containing only PC did not adsorb lipocortins as PC liposomes did not (7). These findings collectively suggest that phospholipid-EGTA columns can be substituted for phospholipid vesicles and enable more precise and quantitative characterization of the Ca\textsuperscript{2+}-dependent phospholipid association of lipocortins.

Occurrence of des 1-12 lipocortin I and des 1-26 lipocortin I was often reported from several organs (5, 44, 47). Intracellular proteases which produce such lipocortin I derivatives have not yet been identified. Our results show cathepsin D and calpain I are possible candidates for the production of des 1-12 lipocortin I and des 1-26 lipocortin I, respectively. In this paper, we first demonstrated using affinity column chromatography (Figs. 7 and 8), vesicle binding analysis (Fig. 9), and equilibrium dialysis (Fig. 10) that the proteolytic modification of both human and porcine lipocortins I at the N-terminal regions leads to a remarkable enhancement of the Ca\textsuperscript{2+} affinity (Fig. 10B). Intact lipocortin I has four Ca\textsuperscript{2+}-binding sites with an apparent \(K_d\) of 90 \(\mu\)M, and these results are in agreement with the studies of Schlaepfer and Haigler (27). Release of the N-terminal tail substantially increased the affinity of lipocortin I for Ca\textsuperscript{2+} (\(K_d\), 18 \(\mu\)M), and the four Ca\textsuperscript{2+}-binding sites were preserved.

Since cathepsin D is a lysosomal enzyme and works under acidic conditions, the degradation of lipocortin I may be attributable to the N terminus of lipocortin I by cathepsin D could be artifactual. Our results suggest that the N-terminal domain of lipocortins regulates the Ca\textsuperscript{2+}/phospholipid binding of the C-terminal core domain in a specific manner for each protein.

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Supplementary Material


to

ENHANCEMENT OF CALCIUM SENSITIVITY OF LIPOCORTIN I IN PHOSPHOLIPID AFFINITY COLUMN CHROMATOGRAPHY AND TREATMENT OF CAMPYLOBACTER WEBS.

EXPERIMENTAL PROCEDURES

Preparation of Active Lipocortin I: A 2000x protein extract was obtained from the control strain of C. jejuni (i.e., 61 strains of bacteria) obtained from TIBS Institute, 200x standard of the bacterial suspension was incubated with 10 nmol of the calcium ionophore A23187 in a total volume of 0.5 ml of 20 mM HEPES buffer, pH 7.5, and incubated for 30 minutes at 37°C. The calcium ionophore was then removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The resulting supernatant was dialyzed against 20 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, and finally concentrated to a final volume of 0.5 ml using a 30,000 MWCO filter. The concentrated sample was then analyzed by SDS-PAGE.

Preparation of Active Lipocortin II: A 2000x protein extract was obtained from the control strain of C. jejuni (i.e., 61 strains of bacteria) obtained from TIBS Institute, 200x standard of the bacterial suspension was incubated with 10 nmol of the calcium ionophore A23187 in a total volume of 0.5 ml of 20 mM HEPES buffer, pH 7.5, and incubated for 30 minutes at 37°C. The calcium ionophore was then removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The resulting supernatant was dialyzed against 20 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, and finally concentrated to a final volume of 0.5 ml using a 30,000 MWCO filter. The concentrated sample was then analyzed by SDS-PAGE.

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Calcium Sensitivity of Lipocortin

RESULTS

Preparation of Phosphohilipid-CPC Column: The amount of phospholipids bound to 1 g of activated CPC ranged 15-40 mg, which was comparable to the value of 43 mg/g calcium ion in the assumption that each phospholipid molecule occupies an area of 22 A

A and the surface area of 1 g of CPC used here was 26

10 m. There was no obvious change in lipid composition between the lipid extracts before and after the phospholipid-containing CPC. Lipocortin were adsorbed to Fas-containing affinity columns in a Ca

-dependent manner and not due to a change of the affinity column. The PC-PS column gave essentially the same results as the PC-PS-chloroform column, and the data obtained by the latter column are presented in the following sections. When 65-100 mg of purified lipocortin was re-applied to the affinity column (1 x 1.5 cm), no detectable amount of lipocortin flowed through the column, and 65-80 mg was recovered by elution with EGTA-containing buffer. The binding capacity of the column (1 x 1.5 cm) for lipocortin had been reused more than 20 times and given consistent results with high reproducibility.

Isolation of Lipocortin I from Human Placenta. When the 30-57 flow-through fraction of human placental crude extract was applied to a phospholipid-CPC column in the presence of Ca

, most of the protein passed directly through the column, and essentially no immunologically detectable amounts of lipocortin I and II were eluted in the flow-through fraction (Fig. 1). Lipocortin I was eluted in a single, sharp peak with buffer B containing 1 mM EGTA (Fig. 1A). When 1 mg of 30-57 flow-through (lanes 1-3) was applied to the column, a major peak was obtained. Electrophoresis of the GTA eluate showed a major band of a 37-kDa protein, minor bands of 34-kDa and 36-kDa proteins, and trace amounts of at least four proteins of 35.3, 34.5, 34, 25 kDa (Fig. 1B, Lane 1). According to their molecular sizes and the results of immunoblotting analysis, the 37-kDa and 38-kDa proteins were identified as lipocortin I and II, respectively. (Fig. 1C and D). Smaller peptides of 34-kDa and 36-kDa proteins were detected by limited proteolysis. The proteins of 34.5 and 35.3 kDa were immunologically related to lipocortin I and II, respectively, and details of these proteins will be described elsewhere.

The mixture of lipocortins I and II was subjected to second affinity chromatography based upon the results of the Ca

 requirement for binding to the phosphohilipid-CPC column. Chromatography was performed with linear gradients of increasing EGTA, and correspondingly increasing GTA, at room temperature. Two major peaks were observed: a large one at 30 mM Ca

 and a small one at 5 mM Ca

 (Fig. 2A). The results of Fig. 2A indicate that lipocortin I binds more tightly and lipocortin II more weakly with Ca

 than with Ca

, which might explain the result of limited proteolysis (Fig. 1D). Chromatography of lipocortin I and II was performed with linear gradients of 150 mM EGTA, and correspondingly increasing GTA, at room temperature. Lipocortin I and II were eluted in a single, sharp peak with buffer B containing 150 mM EGTA (Fig. 2B). Typically, lipocortin I and II were eluted from the affinity column in a single, sharp peak with buffer B containing 150 mM EGTA (Fig. 2B). For each experiment, a mixture of lipocortins I and II was prepared and subjected to affinity chromatography. The proteins of 34.5 and 35.3 kDa were immunologically related to lipocortin I and II, respectively, and details of these proteins will be described elsewhere.

Isolation of Lipocortin I from Porcine Lung: The flow-through fraction of phospholipid-collation chromatography of porcine crude extract was applied to a phospholipid-CPC column. Chromatography was performed with linear gradients of increasing EGTA, and correspondingly increasing GTA, at room temperature. Two major peaks were observed: a large one at 30 mM Ca

 and a small one at 5 mM Ca

 (Fig. 3A). The result of Fig. 3A indicates that lipocortin I binds more tightly and lipocortin II more weakly with Ca

 than with Ca

, which might explain the result of limited proteolysis (Fig. 1D). Chromatography of lipocortin I and II was performed with linear gradients of 150 mM EGTA, and correspondingly increasing GTA, at room temperature. Lipocortin I and II were eluted in a single, sharp peak with buffer B containing 150 mM EGTA (Fig. 3B). For each experiment, a mixture of lipocortins I and II was prepared and subjected to affinity chromatography.

Phosphohilipid-CPC Affinity Chromatography of Porcine Lung Lipocortin I and II. (Fig. 4A) Lipocortin I and II were eluted in a single, sharp peak with buffer B containing 150 mM EGTA and 50% GTA, and 37-kDa lipocortins I and II were applied to the affinity column in the presence of enzyme and eluted with buffer B containing 150 mM EGTA and 50% GTA. The results of Fig. 4B indicate that lipocortin I and II are eluted in a single, sharp peak with buffer B containing 150 mM EGTA and 50% GTA and correspondingly increasing GTA, at room temperature. Lipocortin I and II were eluted in a single, sharp peak with buffer B containing 150 mM EGTA and 50% GTA in the presence of Ca

 (Fig. 4B). Elution was carried out with linear gradients of 0.1 to 0.5 mM Ca

 and 0 to 0.5 mM GTA in 20 mM Tris-Cl pH 7.5 (Fig. 4A). Eluted proteins were analyzed by SDS-PAGE and stained with Coomassie Blue (36). The electrophoresed samples were subjected to immunoblotting analysis using anti-lipocortin I antibody (A) and anti-lipocortin II antibody (B). Samples were lanes 1, 5 µg of the protein/mL; lanes 2, 5 µg of protein/mL; lanes 3, 5 µg of protein/mL; lanes 4, 25 µg of protein/mL; lanes 5, 25 µg of protein/mL; lanes 6, 25 µg of protein/mL; lanes 7, 25 µg of protein/mL.
**Calcium Sensitivity of Lipocortin**

**Fig. 1.** Purification of porcine lung lipocortin using phospholipid-OPS affinity chromatography. Purification was carried out as described in Experimental Procedures. Proteins at each purification step were resolved by SDS-PAGE and stained with Coomassie blue. Lane a, peak fraction containing lipocortin I; lane b, peak fraction containing lipocortin II; lane c, flow-through fraction of phospholipid-OPS column (11 µg); lane d, non-adsorbed proteins eluted with Buffer B containing 2 mM EGTA (3 µg). Samples were run on a Mini-Protean II gel in the presence of 10 µg/ml of protein. Lanes a, crude extract of porcine lung (75 µg of protein); lane b, the flow-through fraction of phospholipid-OPS column (11 µg); lane c, non-adsorbed proteins eluted with Buffer B containing 2 mM EGTA (3 µg). The numbers on the left side represent the molecular masses of standard proteins.

**Fig. 4.** Comparison of porcine lipocortin I derivatives on SDS-PAGE (A) and amino acid sequence analysis (B). Porcine lung lipocortin I and its derivatives were prepared by SDS-PAGE and amino acid sequence analysis as described in Experimental Procedures. Lane a, 17-kDa lipocortin I purified by affinity chromatography and chromatofocusing; lane b, the product after treatment of lipocortin I with carboxypeptidase Y; lane c, the product after treatment of 17-kDa lipocortin I with carboxypeptidase Y; and lane d, 35.5-kDa lipocortin I purified according to the method of Do et al. (44). Molecular masses of proteins are given in kDa. The bottom line in panel B represents the predicted N-terminal sequence of human lipocortin I (11).

**Fig. 8.** Affinity column chromatography of porcine lung lipocortin I, des 1-12 lipocortin I, and des 1-26 lipocortin I with 50% SDS gradient. Lane 1, 17 kDa; lane 2, 18.5 kDa; lane 3, 34 kDa; lane 4, 35.3 kDa; lane 5, 37.7 kDa. Amino acid sequence analysis revealed that the gradient was applied to a phospholipid-OPS column and aed with a decreasing Ca^2+ gradient at room temperature (A). Eluted proteins were subjected to SDS-PAGE and stained with Coomassie Blue (B). Molecular masses of proteins are expressed in kDa.