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PURIFICATION OF CALCIUM-SENSITIVE REGULATORY PROTEIN OF PLATELETS WHICH INHIBITS THE GELATION OF ACTIN

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Ca^{2+}-sensitive regulatory protein of human platelets which inhibits the gelation of actin was purified by DEAE-Sepharose and an affinity column using actin as a ligand. The protein was a single polypeptide chain with an average molecular weight of 90,000 and it bound to actin and inhibited its gelation at concentration from 10^{-6}-10^{-7}M of free calcium. Since the protein existed in the form of a complex with actin even though at concentration lower than 10^{-7}M of free calcium, binding and dissociation of actin and the protein appeared to be dependent on the concentration of free calcium, and complete dissociation was not seen.

Actin is an important protein not only in the contraction of muscle cells but also in the motion of non-muscle cells(1). As in the motion of non-muscle cells, locomotion, secretion, endocytosis and so on, actin filaments were formed in the periphery of the cytoplasm(2), thus the gel-sol transformations of actin is thought to be essential factor of these phenomena. Physiological concentration of calcium affects and regulates these phenomena but purified actin itself has no effect of calcium in polimerization(3). Therefore in non-muscle cells some factors must be involved in the regulation of the gel-sol transformation of actin in the presence of physiological concentration of calcium. Some of these factors were reported as gelsolin from macrophage(4,5), villin from microvilli(6), fragmin from plasmodia of myxocete(7), and actinogelin from

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Ehrlich tumor cells(8). Also in platelets, actin has an important role in the appearance of aggregation or adhesion of platelets(9), and these functions of platelets are greatly affected by the calcium concentration(10,11).

In this work, we show a rapid and efficient purification method of actin-interacting protein, which enables the purification of calcium-activated regulatory protein that inhibits the gelation of actin.

MATERIALS AND METHODS

Preperation of Human Platelets Human peripheral blood containing anticoagulant was centrifuged at 140g for 10min and the supernatant was recentrifuged until the pellet lost almost all of its red color. Thus the supernatant was centrifuged at 8,000g for 10min, and the pellet of platelets was washed 2 times with saline, about 15g of packed platelets were obtained from 7L of blood.

Preperation of Sepharose-Protein Conjugates Approximately 500mg of G actin was prepared as previously described(12). The protein was coupled to CNBr activated Sepharose 4B as described by Axen et al(13) with slight modification, 15g of CNBr activated Sepharose 4B (Pharmacia Fine Chemicals) was washed with 1L of 1mM HCl for 15min, and then washed with 1L of 0.1M $NaHCO_3$ buffer pH8.3 (coupling buffer), and resuspended with the same buffer. G actin solution dialyzed against coupling buffer was added to the Sepharose solution and shaken for 2 hours at room temperature. The actin and Sepharose solution was washed with 1L of 0.2M Glycine pH8.0, resuspended with the same buffer and shaken for 2 hours at room temperature. The resultant solution was washed with 250mL of 0.1M CH_3COOH buffer pH4.0 and then with 250mL of coupling buffer, the washing was repeated 3 times. With this actin binding Sepharose solution, a column 3cmx15cm was obtained.

Purification of Actin Gelation Inhibitor Approximately 15g of packed platelets were homogenized by a teflon homogenizer with 5 volume of ice-cold homogenizing solution containing 0.34M sucrose, 5mM DTT, 20mM imidazole-HCl, 5mM EGTA, 5mM ATP, and 0.25mg/mL soy bean trypsin inhibitor pH7.4. The homogenate was centrifuged at 20,000g for 20min and the supernatant, designated S_1 was collected. To S_1, KCL and $MgCl_2$ were added to a final concentration of 0.1m and 5mM, respectively. After standing for 1 hour at room temperature, the solution was recentrifuged at 20,000g for 20min. The resulting supernatant, designated S_2, was collected, and dialyzed over night against 1mM EGTA, 1mM DTT, 0.5mM ATP, and 1mM imidazole-HCl, pH7.8 (buffer A). The dialyzed S_2 was then applied onto a DEAE Sepharose column (2.6x20cm) which was equilibrated with 0.1M KCL in buffer A. After washing with 250mL of buffer A and then with 250mL of 0.1M KCl in buffer A, the column was eluted with 500mL of KCl linear gradient (0.1M-0.4M KCl in buffer A). The eluates which contain the activity of calcium sensitive actin gelation inhibitor were collected and designated S_3. S_3 was dialyzed against 1mM $CaCl_2$, 0.1M KCl, and 20mM imidazole-HCl,

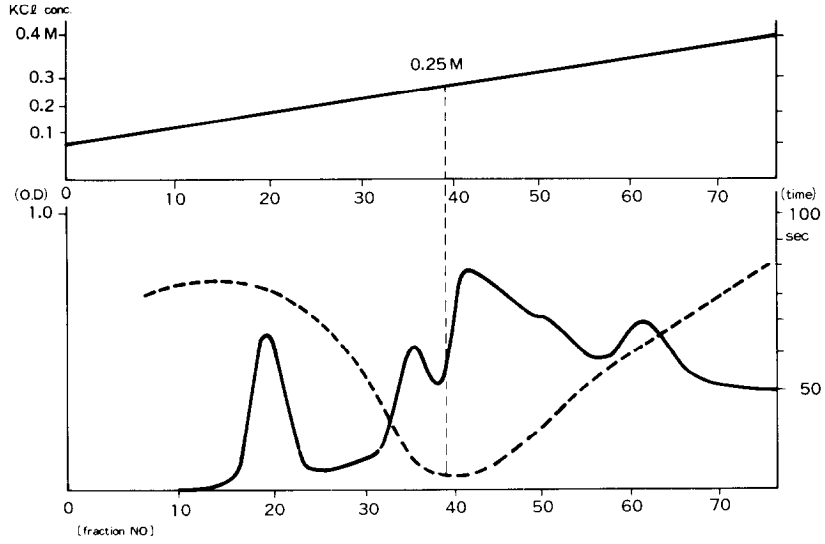

Fig. 1. Ion exchange chromatography of S_2. A column of DEAE sepharose CL 6B (2.6x20cm) was equilibrated with 0.1M KCl in buffer A (1mM EGTA, 1mM DTT, 0.5mM ATP, and 9mM imidazole-HCl, pH7.8). The column was washed with 0.1M KCl in buffer A, then eluted with linear gradients of 0.1M-0.4M KCl in buffer A. —— shows the absorbance at 280nm, ---- shows actin gelation inhibitory activity (sec).

pH7.2 (buffer B), and then applied onto the affinity column using actin as ligand (3x10cm) and equilibrated with buffer B. After washing with excess of buffer B, the column was eluted with 10mM EGTA in buffer B, and the protein containing eluates were collected, and designated S_4. S_4 was applied onto the DEAE Sepharose column (1.8x10cm) equilibrated with 0.1M KCl in buffer A, washed with the same solution, and then eluted with 200mL of KCl linear gradient (0.1M-0.6M KCl in buffer A), the eluate was designated S_5.

Assay of Ca^{2+} sensitive Actin Gelation Inhibitory Activity Actin gelation inhibitory activity was measured by a modified method of Maclean's falling ball method(14). 30μL of the protein solution dialyzed against buffer B, 20μL of G actin solution (protein concentration 2.7-4.7mg/mL), and 100μL of buffer B were mixed and drawn into a capillary tube (Drumond 150μL) sealing one end with clay, and fixed at 25° from the horizontal line. After 10min, a steel ball (ø0.7mm) was placed into the capillary and the time needed for the ball to travel 10cm down the solution in the capillary tube was measured. As a control, measurements were also done with 20μL of G actin solution and 130μL of buffer B.

SDS Polyacrylamide Gel Electrophoresis The samples were electrophoresed as previously described(15), using gradient gel (5-15%). The molecular weight of the samples were determined by the electrophoresis patterns of the standard proteins: phosphorylase (94,000), bovine serum albumin (67,000), carbonic anhydrase (30,000), soy bean trypsin inhibitor (20,100), and α lactalbumin (14,400).

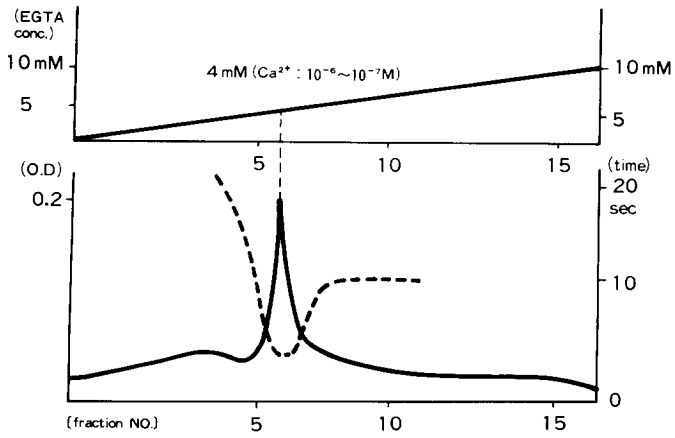

Fig. 2. Affinity chromatography of S_3. An affinity column (3x10cm) using actin as a ligand was equilibrated with buffer B (1mM $CaCl_2$, 0.1M KCl, and 20mM imidazole-HCl, pH7.2). The column was washed with buffer B, then eluted with linear gradients of 0-10mM EGTA in buffer B. —— shows the absorbance at 280nm, ---- shows actin gelation inhibitory activity (sec).

RESULTS AND DISCUSSION

The elution profile of the first chromatography by DEAE Sepharose CL 6B is shown in Figure 1. The maximum gelation inhibitory activity was eluted at the KCl concentration of about 0.25M. Fractions containing the gelation inhibitory activity (less than 20seconds) was collected and dialyzed against buffer B. The second chromatography by actin binding affinity column revealed a single peak at 0.D 280nm (Figure 2), which showed good correlation with gelation inhibitory activity. The free calcium concentrations at which the peak was eluted were 10^{-6}- 10^{-7}M calculated by using $CaCl_2$ EGTA containing gradient buffer. This findings showed that the actin as a ligand and the actin gelation inhibitor were bound at calcium concentrations greater than 10^{-6}M, but then dissociated at concentrations below 10^{-7}M. This concentration range (10^{-6}-10^{-7}M) is similar to intracellular free calcium concentration. The SDS polyacrylamide gel electrophoresis pattern of this sample showed three bands (molecular weight: 90,000, 75,000 and 43,000) as calculated from the standard proteins. The band

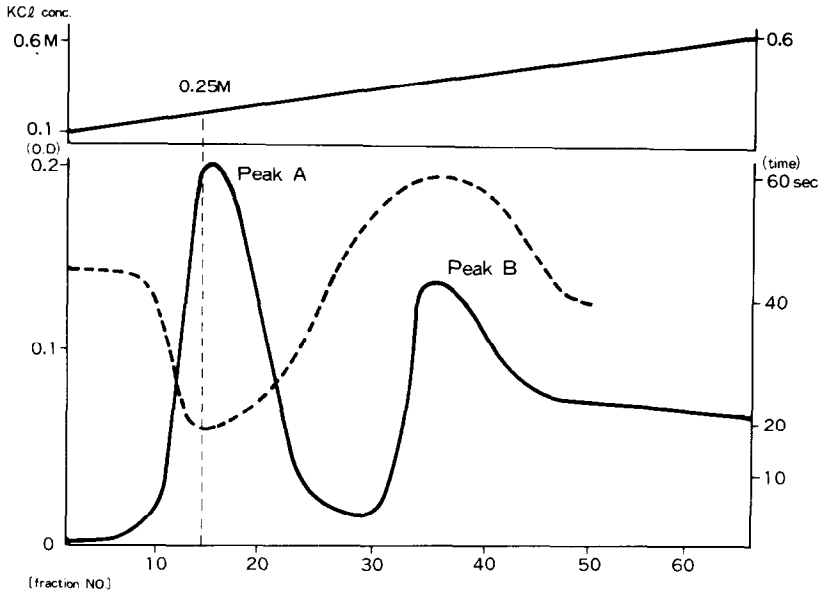

Fig. 3. Ion exchange chromatography of S_4. A column of DEAE Sepharose CL 6B (1.8x10cm) was equilibrated with 0.1M KCl in buffer A (1mM EGTA, 1mM DTT, 0.5mM ATP, and 9mM imidazole-HCl, pH7.8). The column was eluted with linear gradients of 0.1M-0.6M KCl in buffer A. —— shows the absorbance at 280nm, ---- shows actin gelation inhibitory activity (sec).

of 43,000 had the same molecular weight of actin, indicating that the band originated from the actin as a ligand or the actin from platelets. However, there is a possibility that the band originates from a subunit of the gelation inhibitor, thus the sample S_4 was rechromatographied using a DEAE Sepharose CL 6B column, the elution profile of which is shown in Figure 3. Two peaks were obtained at the absorbance at 280nm, the first peak was designated peak A and second peak, peak B. Peak A was eluted at about 0.25M KCl and had gelation activity, the peak B had less activity than the control. SDS polyacrylamide gel electrophoresis of peak A and peak B are shown in Figure 4. Peak A consisted of two bands, 90,000 and 43,000, peak B had only the band of 43,000. These results of the activity and SDS polyacrylamide gel electrophoresis pattern indicated that the gelation inhibitor was present in peak A,

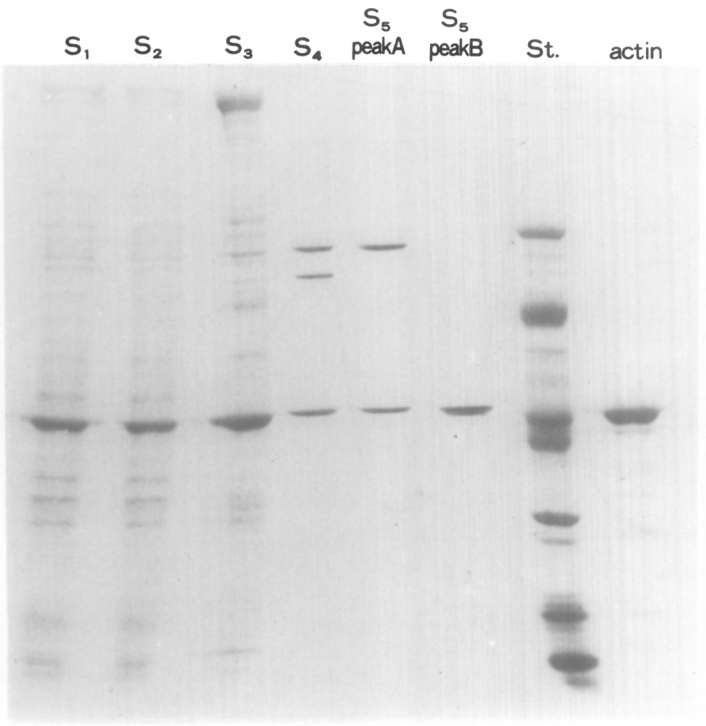

Fig. 4. SDS-polyacrylamide gel electrophoresis of fractions obtained during the purification of actin gelation inhibitor (gradient gel of 5-15%) S_1: platelet extract, S_2: sample after the treatment of 0.1M KCl, S_3: DEAE-sepharose fraction, S_4: affinity chromatography fraction, S_5: rechromatorgraphy with DEAE-Sepharose. S_5-peak A: first eluted peak. S_5-peak B: second eluted peak. LMW: standard proteins (phosphorylase, bovin serum albumin, carbonic anhydrase, soy bean trypsin inhibitor, and α lactalbumin).

and peak B was comprized of actin which originated from the column or platelets. The possibility that the band of 43,000 was not a subunit of the gelation inhibitor but of actin was strongly suggested from the densitometrical pattern of SDS polyacrylamide gel electrophoresis of S_4, and S_5 peak A as shown in Figure 5. By affinity chromatography, three bands were obtained on SDS polyacrylamide gel electrophoresis, the bands had molecular weight of 90,000, 75,000, and 43,000. By rechromatography with DEAE Sepharose, after washing the column, the band of 75,000 had almost disappeared, but the bands of 90,000 and 43,000 remained. The subsequent ratio of bands of 90,000 and 43,000 in S_4 and S_5 peak A were reversed, demon-

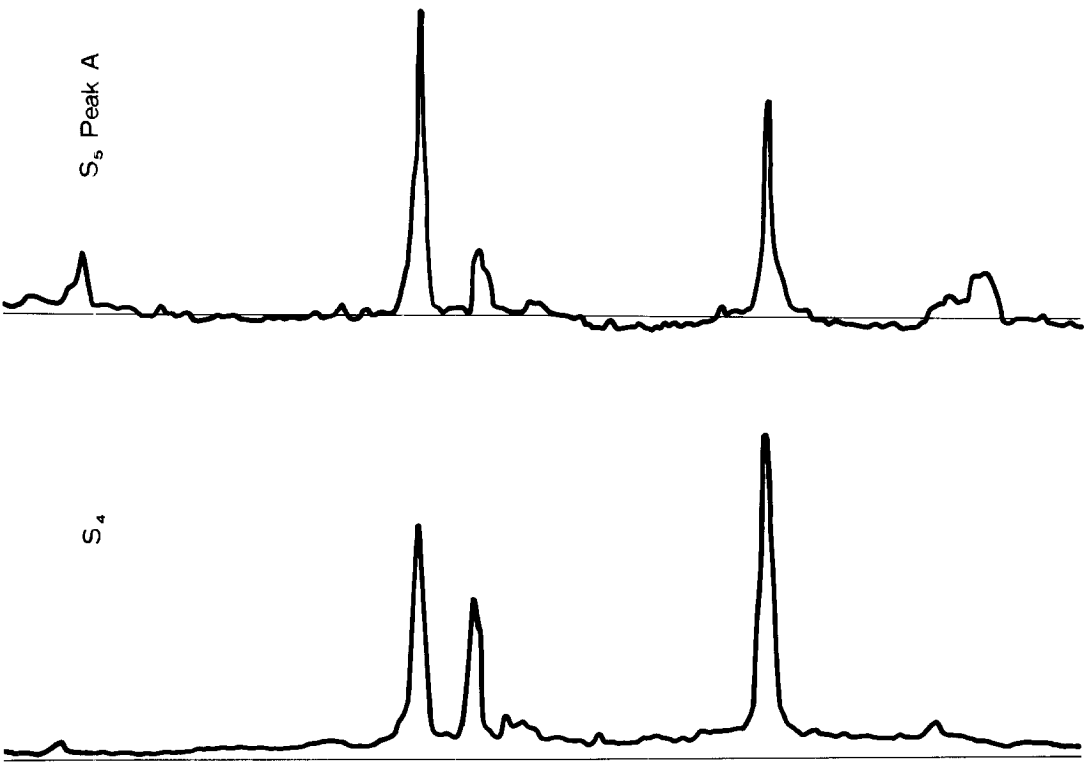

Fig. 5. Densitometrical patterns of SDS polyacrylamide gel electrophoresis of S_4 and S_5 peak A. (gradient gel of 5-15%).

strating that the band of 43,000 was not a subunit of the gelation inhibitor. From these results, we concluded that the band of 90,000 is the protein that inhibits the gelation of actin. However, the gelation inhibitor of band of 90,000 and actin of band of 43,000 could not be separated even though peak A and peak B were completely separated upon rechromatography of DEAE Sepharose with 1mM EGTA containing buffer. In addition, the gel filtration by Sephadex G 200 with 1mM EGTA containing buffer of peak A revealed a single protein peak with a molecular weight calculated at about 120,000. These results suggested that the gelation inhibitor and actin were partially bound even at low concentration of free calcium, and with increasing concentration of free calcium the binding of actin and gelation inhibitor increased. This supposition

must be clarified by further investigation. After the SDS polyacrylamide gel electrophoresis of the peak A, the gel band of 90,000 was then sliced, and extracted with buffer A showed a single band. This sample had the activity of gelation inhibition of actin, and by gel filtration with Sephadex G 200, the molecular weight was determined to be about 90,000. Thus the gelation inhibitor is a monomeric protein with a molecular weight of about 90,000. These results differ from other actin gelation inhibitors, gelsolin from macrophage(4,5) has two subunits both with a molecular weight of 90,000, fragmin(7) is a monomer of 50,000. Moreover, the inhibitor we investigate is different from caldesmon(16) in calcium sensitivity and molecular weight, however it resembles villin from microvilli (6) which is a monomer of 95,000. Investigation is needed to characterize this calcium sensitive actin gelation inhibitor.

REFERENCES

1. N. Shibata, N. Tatsumi, N. Senda, (1972) Biochem. Biophys. Acta. 256, 565-576.
2. Reaven, E.P. & Axline, S.G. (1973) J. Cell Biol. 59, 12-27.
3. Brotshi, E.A., Harwig, J.H. & Stossel (1978) J. Biol. Chem. 253, 8988-8993.
4. Helen L. Yin & Thomas P. Stossel (1979) Nature. 281, 583-586.
5. Helen L. Yin & Thomas P. Stossel (1980) J. Biol. Chem. 255, 9490-9493.
6. Anthony Bretcher & Klaus Weber (1980) Cell. 20, 839-847.
7. Takayuki Hasegawa, Sho Takahashi, Hiroshi Hayashi, & Sadashi Hatano (1980) Biochemistry. 19, 2677-2683.
8. Naotoshi Mimura & Akira Asano (1979) Nature. 282, 44-48.
9. Cohen, I. & Cohen, C.A. (1972) J. Mol. Biol. 68, 383-387.
10. Betlix-Gill and, M. & Luscher, E.F. (1961) Biochem. Biophys. Acta. 49, 536-547.
11. Le Breton, G.C., Sandler, W.C. & Feinbery, H. (1976) Thromb. Res. 8, 477-485.
12. James A. Spudich, & Susan Watt (1971) J. Biol. Chem. 246, 4866-4871.
13. Axen, R., Porath, J. & Ernback, S. (1967) Nature. 214, 1302-1304.
14. Susan D. Maclean-Fletcher & Thomas D. Pollard (1980) J. Cell Biol. 85, 414-428.
15. F. William. Studier (1973) J. Mol. Biol. 79, 237-248.
16. Kenji Sobue, Yoshihiko Muramoto, Masataka Fujita & Shiro Kakiuchi (1981) Proc. Natl. Acad. Sci. USA. 78, 5652-5655.