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Development of affinity chromatography using a bioactive peptide as a ligand

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Abstract—By repeatedly introducing hydrophilic polyethylene glycol (PEG) spacer (**2**) onto affinity resin bearing a bioactive peptide (1/2 secretory leukocyte protease inhibitor, 1/2SLPI) as a ligand, the adsorption of nonspecific binding proteins was effectively reduced and the purification efficacy of elastase, which is one of the target molecules for 1/2SLPI, from a protein mixture was improved. Moreover, using this resin, we also successfully detected L-plastin, as an endogenous target molecule for SLPI, from HL-60 cell lysate.

1. Introduction

Since the decoding of the human genome, drug discovery techniques utilizing genomic information, the so-called genome-based drug discovery method, have attracted much attention as a strategy for new drug discovery. The identification and acquisition of new drug target proteins is a key challenge in genome-based drug discovery, and affinity chromatography is gaining renewed attention as a technique for discovering such target proteins.[1,2] Much interest in particular has recently been drawn to the technique of low molecular weight compound immobilized affinity chromatography. However, improvements in affinity chromatography are urgently needed to enhance its convenience and increase the purification efficiency of target proteins for the ligands by reducing the adsorption of nonspecific binding proteins to the affinity resins.[3,4] AffiGel™,[5] which is made of agarose, has long been used as for affinity chromatography as a resin, but improvement of its hard handling and, physical and chemical stability has proved to be problems. An affinity resin that represents a solution to these problems is the polymethacrylate resin TOYOPEARL™.[6] While the above disadvantages of the affinity resin AffiGel™ have been addressed, a new challenge remains to be solved, the copious adsorption of nonspecific binding proteins to its surface. Recently, Tanaka et al. developed a basic technique in which the adsorption of nonspecific binding proteins is reduced in a low molecular weight ligand immobilized affinity chromatography.[7] We investigated the application of this technique to bioactive peptides, the reason being that affinity chromatography is yet to be optimized as a method for detecting target ligands to bioactive peptides. In this report, we show a technique for efficiently purifying the target protein from a tissue lysate and capturing ability of the endogenous target protein from a cell lysate.

One of the most attractive and significant bioactive peptides is secretory leukocyte protease inhibitor (SLPI). SLPI is reported to have diverse bioactivities, including not only protease inhibitory,[8] but also anti-inflammatory[9] and fibrosis inhibitory activity.[10] However, the elucidation of its target proteins remains an unresolved issue. As well, SLPI has a characteristic structure. X-ray crystallographic analyses have shown that SLPI comprises two separate domains of similar architecture, and that each domain has four disulfide bonds (Fig. 1A).[11] Focusing attention on this sequence and the structural homology, a recombinant C-terminus half SLPI (Arg58-Ala107: 1/2SLPI) was prepared (Fig. 1B), and its bioactivity was investigated. This investigation confirmed that both 1/2SLPI and SLPI similarly have protease inhibitory activity,[12] and 1/2SLPI is also expected to have diverse physiological activities.

Keywords: Nonspecific binding proteins; Affinity chromatography; Hydrophilic spacer; Secretory leukocyte protease inhibitor.
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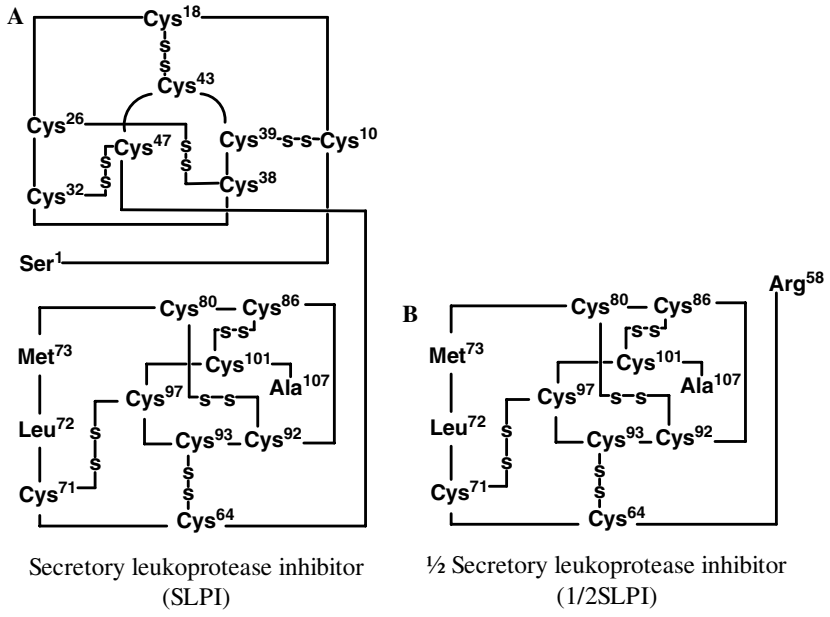
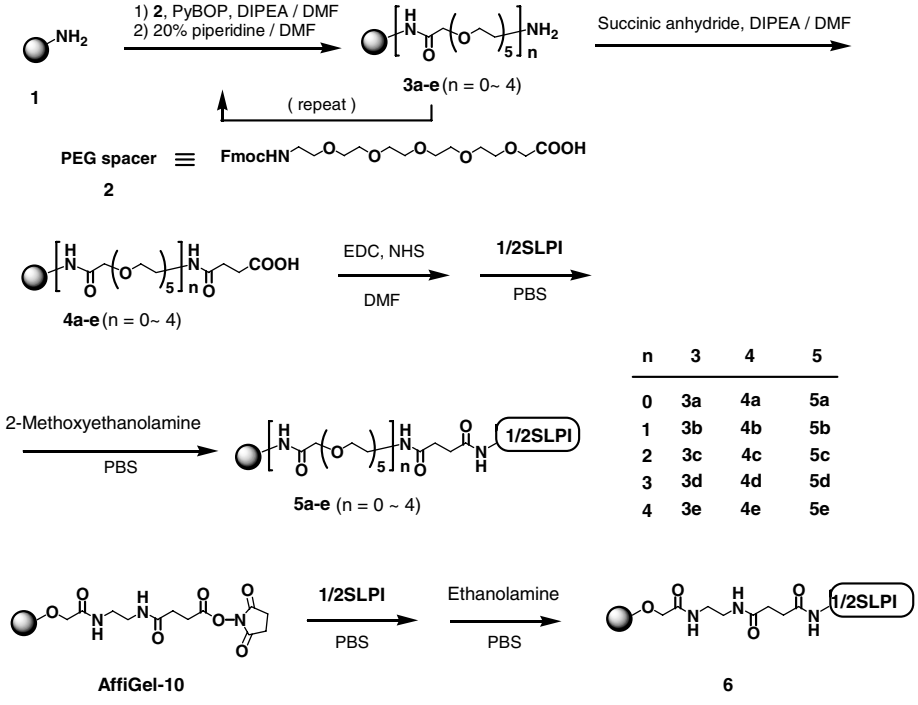


Figure 1. Structure of SLPI (A) and 1/2SLPI (B). 1/2SLPI is the divided C-terminal part at 58-position of SLPI.

For the above reasons, 1/2SLPI was selected as the bioactive peptide to be immobilized on an affinity resin (TOYOPEARL™), and a method for efficiently extracting the target molecule was investigated.

2. Preparation of affinity resin

To optimize the affinity chromatograph technique, bioactive peptide 1/2SLPI immobilized affinity resins (TOYOPEARL™) (**1**) were prepared as follows (Scheme 1). TOYOPEARL™ is physically and chemically stable, and handles easily however, the adsorption of nonspecific binding proteins due to its surface hydrophobicity remains the problem to be overcome. Recently introduction of hydrophilic PEG spacer (**2**) is reported to be very effective to reduce adsorption of nonspecific binding proteins. In such a study a low molecular compound was used as a ligand,[7] so we examined the introduction of hydrophilic PEG spacer (**2**) between TOYOPEARL™ surface and 1/2SLPI. As shown in Scheme 1, PEG spacer (**2**) was reacted with terminal amino groups on



Scheme 1. Preparation of affinity resins bearing 1/2SLPI with or without hydrophilic PEG spacer (**2**).

TOYOPEARL™ using condensation reagents (PyBOP/DIPEA). Unreacted amino groups on the resin surface were capped by the reaction with acetic anhydride, after that Fmoc protecting groups of amino groups were removed to be confirmed the introduction of PEG spacer on the resin by Ninhydrin test.

By repeatedly introducing this PEG spacer, affinity resins with PEG spacers of differing lengths were prepared (**3**). Usually peptide or protein is immobilized as a ligand on solid support, which has carboxylic acid, by use of active ester. This methodology, using active ester, is very useful because chemical modification hardly occurs on a ligand and the native bioactivity of a ligand does not diminish. So we selected this active ester method to immobilize 1/2SLPI on affinity resin. To apply this method to 1/2SLPI on TOYOPEARL™, carboxylic acid was introduced at the terminal of the linker of affinity resin using succinic anhydride. This amide bond formation reaction proceeded quantitatively and resin bearing carboxylic acid (**4**) was obtained. In the case of immobilization of the ligand to solid support with active ester method, activation of carboxylic acid and amide bond formation reaction are usually performed in an aqueous solution however, *N*-hydroxysuccinimide and resulting active ester are not stable in such an aqueous solution. So if activation of carboxylic acid and amide bond formation reaction were performed in an aqueous solution, high reaction rate would not be expected. On the other hand hydroxysuccinimide ester is very stable in aprotic organic solvent. So chemical modification of terminal functional group to active ester formation on resin was performed in aprotic organic solvent, and in this study DMF was selected because of the chemical stability of TOYOPEARL™. Following amide bond formation with 1/2SLPI was carried out in phosphate buffer solution after quick change of the solvents. And after that remaining active ester groups were capped by excess amount of 2-methoxyethylamine to afford 1/2SLPI immobilized resins (**5a**–**e**). Meanwhile, to confirm the performance of the affinity resins, 1/2SLPI immobilized AffiGel (**6**) was also prepared as a reference standard.[5] In this case, AffiGel-10™, which has active ester residues, was used. By use of AffiGel-10™, 1/2SLPI was immobilized both AffiGel and TOYOPEARL™ under almost the same condition. Ligand immobilization was carried out according to the manufacturer's instructions.

3. Results and discussion

3.1. Reduction of nonspecific binding proteins on affinity resin by introduction of 2

To demonstrate the differences in extraction efficiency of a target protein using these prepared affinity resins, we examined a binding study using a protein mixture. Lysate prepared from rat brain was used as a protein mixture.[7,13] As well, in order to compare extraction efficiency of target molecules, a binding study was performed after adding a small amount of elastase to this protein mixture beforehand. The results of protein binding test on affinity resins were shown in Figure 2. Lanes

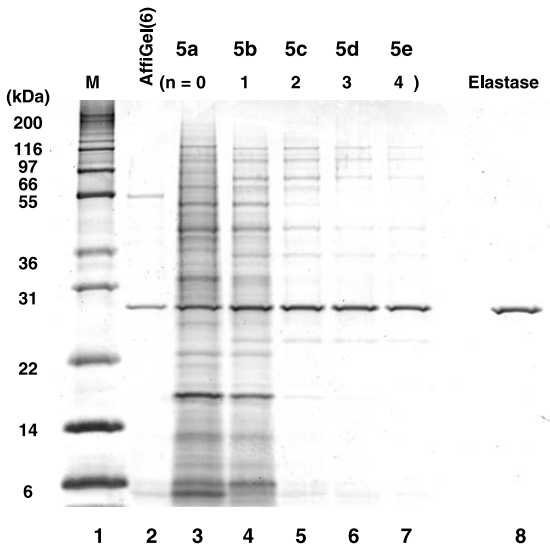


Figure 2. Binding proteins on affinity resins bearing 1/2SLPI with or without PEG spacer (**5a**–**e**, and **6**).

2 and 3 in Figure 2 show the adsorbed proteins to both resins AffiGel (**6**) and TOYOPEARL (**5a**), in which 1/2SLPI was immobilized directly on each resin. Elastase which is one of the target molecules for 1/2SLPI, was obtained by both resins, but the ability of TOYOPEARL to capture the target molecule was superior. This may be because TOYOPEARL™ has more reaction points on the resin surface into which the ligand can be introduced than AffiGel. In contrast, abundant protein adsorption was observed on the resin surface of TOYOPEARL. Typically, polymethacrylate resin have greater adsorption of nonspecific binding proteins mediated by hydrophobic interactions with the surface, as compared with agarose gels.[7] We investigated the effect of introducing hydrophilic PEG spacers to the methacrylate resin surface, using a bioactive peptide as a ligand.

Then we compared the protein adsorption to the surface of affinity resins **5a**–**e** (Scheme 1) and AffiGel **6**, using a protein mixture that included a small amount of elastase. The results are shown in lanes 4–7 in Figure 2. It is obvious that the capturing efficiency for the target protein elastase was maintained and the adsorption of nonspecific binding proteins was dramatically reduced by the repeated introduction of PEG spacer. And the effect was reached maximum at **5d**, which was introduced PEG spacer three times. Thus, despite an absence of changes in the extraction performance for the target protein, the proteins which were reduced to adsorb to the resin as a result of the increased hydrophilicity of the resin surface are considered to be hydrophobic interaction mediated nonspecific binding proteins.

3.2. Quantitative analysis of capturing ability of target protein and reduction effect of nonspecific binding proteins

Next, to quantitatively evaluate the extraction efficiency of each resin for elastase, the relative intensity of elastase in each lane and the purification yield were calculated by image analysis. The results of these calculations

are shown in Figure 3. This result let us confirm that the extraction performance of TOYOPEARL™ for elastase was approximately constant, irrespective of differences in the number of PEG spacers introduced (Fig. 3A). In contrast, it was demonstrated that the purification performance for elastase was significantly improved by the repeated introduction of PEG spacers. Compared with no PEG spacer (**5a**), the purification efficiency of elastase was approximately 4-fold higher when PEG spacer had been introduced four times (**5e**). Moreover, via the introduction of PEG spacers onto the TOYOPEARL, the purification efficiency for the target protein elastase was superior to that of AffiGel. These results were probably achieved because the hydrophilic PEG spacers introduced onto the TOYOPEARL™ resin increased the hydrophilicity of the resin surface, and effectively reduced the adsorption of hydrophobic nonspecific binding proteins. Another reason may be the fact that the amount of 1/2SLPI introduced per unit volume of TOYOPEARL is greater than that of AffiGel.

3.3. Detecting ability of endogenous target protein with 1/2SLPI immobilized resins

Next, using the prepared affinity resin **5a** and **5e**, we tested the efficacy of reduction of nonspecific protein binding by introduction of PEG spacer onto TOYOPEARL and capturing ability of target molecules, L-plastin, for 1/2SLPI from a native protein mixture. L-Plastin was reported as one of the target proteins for SLPI and found at about 62 kDa in a gel after SDS–PAGE.[14] Furthermore L-plastin is also confirmed inflammatory related molecule.[14] In this investigation a cell lysate of HL-60, which is a cell line derived from human leukemia cells, was selected as a native protein source because it is a lymphocyte lineage cell line, and is therefore considered to be suited to capture inflammatory related molecules. To detect specific binding proteins for 1/2SLPI, competition study was also performed concurrently with the binding study. That is, binding proteins on these resins' surfaces were compared, with or without free 1/2SLPI added beforehand to the cell lysate. And then the bands on SDS–PAGE gel were compared with differences of binding proteins on 1/2SLPI immobilized resins. As shown in Figure 4A, lots of nonspecific biding proteins to **5a** (lanes 2 and 3) were observed, and specific binding proteins were not able to be distinguished by comparison of these lanes. The effective reduction of such nonspecific binding proteins by repeated introduction of PEG spacer was also observed in this experiment as same as the case of rat brain lysate. And in the vicinity of 62 kDa on lanes 4 and 5 in Figure 4A, the bands indicated by the arrow were detected with apparently different intensity. In this way by repeated introduction of PEG spacer to TOYOPEARL target protein detecting ability was also drastically improved using HL-60 cell lysate. At the same time we compared target protein capturing ability with resin **5e** and **6**, AffiGel. By comparison with bands indicated arrow in lanes 6 and 7 in Figure 4A, the specific binding protein was also detected with resin **6**. But the intensity of focused protein on lane 6 was weak compared with lane 4 in Figure 4A. This result also showed the endogenous target protein purifying efficacy of TOYOPEARL repeatedly introduced PEG spacer **5e** from native protein source is also supposed to be superior to AffiGel **6**.

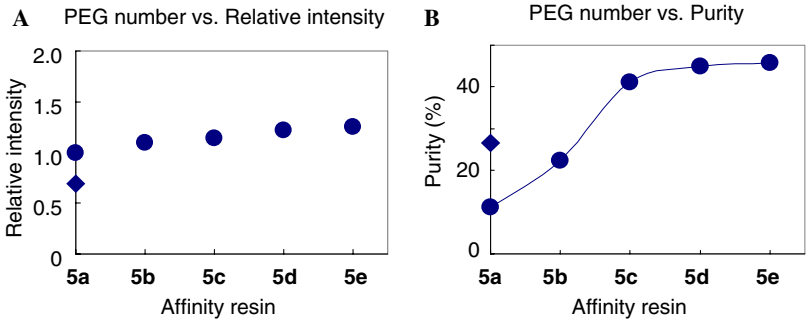


Figure 3. (A) Plots of relative intensity of elastase and (B) purity in each lane with numbers of hydrophilic spacer **2**. (●) and (♦) show TOYOPEARL and AffiGel, respectively. The amount of elastase and purity of elastase in each lane were measured by a GS-800 Calibrated Imaging Densitometer (BIO-RAD, software; Quantity-One-4.4.0).

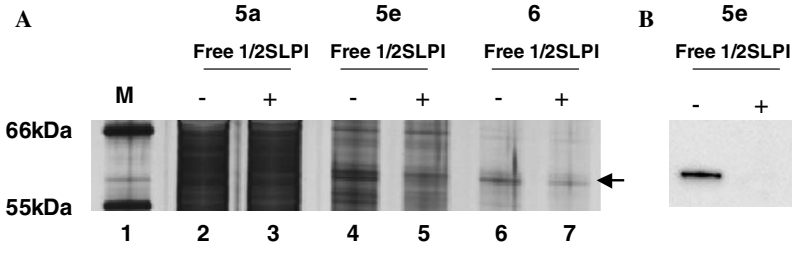


Figure 4. Binding proteins on **5a**, **5e**, and **6** focused around 62 kDa. (A) Results of competition assay using **5a**, **5e**, and **6**. (B) Western blot study using anti-L-plastin antibody on **5e** with competition assay samples.

In addition to above shown target protein purification efficacy, TOYOPEARL has some experimental merits besides chemical and physical stability. That is, compared with AffiGel, TOYOPEARL is easily spin-downed by simple centrifugation and easily distinguishable from solution. So by using TOYOPEARL it is not necessary to consume much time in wash process, and to be suffering loss of affinity resin in all experimental steps.

Eluates obtained from the competition and binding assay on **5e** were subjected to Western blot analysis and the results are as shown in Figure 4B. This result clearly showed that the focused band around 62 kDa was L-plastin.[15] Furthermore we confirmed L-plastin bound with 1/2SLPI as same as SLPI.[14]

4. Conclusions

We investigated the application of an affinity resin, which is composed of TOYOPEARL™, PEG spacer, and bioactive peptide (1/2SLPI) (Fig. 1) as a ligand. The result of the extraction efficiency of the target molecule (elastase) from a protein mixture confirmed that hydrophilic PEG spacers and the ligand had been effectively introduced to the resin surface, utilizing the chemical and physical stability of TOYOPEARL™. Moreover, by making the resin surface highly hydrophilic via repeated introduction of hydrophilic PEG spacers (Scheme 1), adsorption of nonspecific binding proteins was effectively reduced, while the capturing ability of the target molecule (elastase) was maintained (Figs. 2 and 3). Furthermore, the purification efficacy of an endogenous target protein, L-plastin, from a native protein source by introduction of PEG spacer between TOYOPEAL and a bioactive peptide, 1/2SLPI, was also observed (Fig. 4). This methodology is not only applied to small molecular weight compounds but also other bioactive proteins or peptides, and is expected to be a general technique for target finding.

5. Experimental

The following abbreviations were used: buffer A, an aqueous solution of 25 mM Tris–HCl (pH 7.4) and 0.25 M sucrose; DMF, dimethylformamide; BB, bromophenol blue; EDC·HCl, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride; DIPEA, *N*,*N*-diisopropylethylamine; NHS, *N*-hydroxysuccinimide; 2-ME, 2-mercaptoethanol; PEG, poly(ethylene glycol); PBS, phosphate-buffered solution; PyBOP, bromotris(pyrrolidino)phosphonium hexafluorophosphate; rt, room temperature; SDS, sodium dodecylsulfate; EDTA, ethylenediamine tetraacetate; TNE buffer, an aqueous solution of 20 mM Tris–HCl, 150 mM NaCl, and 2 mM EDTA.

5.1. Materials

All reagents were used as received. TOYOPEARL was obtained from TOSHO, AffiGel-10 was purchased from Bio-Rad. PyBOP and EDC·HCl were obtained from Watanabe Chemical Industries, Ltd DIPEA, DMF, 2-methoxyethylamine, ethanolamine, and piperidine were purchased from Aldrich. Compound **2** was synthesized as described previously.[7] A mouse anti-L-plastin monoclonal antibody was obtained from Lab Vision-Neo-Markers, Fremont, CA. Anti-mouse monoclonal antibody and Detecting reagent ECL Plus™ were obtained from Amersham Biosciences Corp. Recombinant 1/2SLPI was obtained as described previously.[12] Protease inhibitor Complete™ was obtained from Roche. Nonidet P-40 was purchased from Nacalai tesque.

5.2. Representative procedure for introduction of hydrophilic space 2 on resins (3)

A mixture of **2** (42 mg, 0.08 mmol), TOYOPEARL (200 μl, 0.02 mmol), PyBOP (52 mg, 0.1 mmol), DIPEA (68 μl, 0.20 mmol), and DMF(1 ml) was shaken at rt for 16 h. After filtration, the resin was washed with DMF five times. The reaction ratio was determined by Ninhydrine test (90%). After washing the resin with DMF, 1 ml of a mixture (acetic anhydride/DMF = 1:4) was added for acetyl capping of remaining amino groups. The reaction mixture was shaken at rt for 2 h, and the resin was washed five times with DMF after removal of the reaction solution.

This Fmoc resin was mixed with 1 ml of a mixture (piperidine/DMF = 1:4) at rt for 2 h. After filtration, the resin was washed with five times DMF to afford the objective resins bearing the hydrophilic spacer (**3**).

5.3. Introduction of carboxylic acid at the terminal of resins (4)

A mixture of **3a** (0.2 ml), succinic anhydride (8 mg, 0.08 mmol), DIPEA (1.5 μl, 0.01 mmol), and DMF (1 ml) was shaken at rt for 15 h. After filtration, the resin was washed five times with DMF to afford the objective resin bearing carboxylic acid at the terminal (**4a**). The reaction ratio was determined by Ninhydrine test (quant.).

The other resins bearing carboxylic acid at the terminal (**4b**–**e**) was prepared in a same manner.

5.4. Preparation of 1/2SLPI immobilized resins (5)

A mixture of **4a** (0.2 ml), EDC·HCl (8 mg, 0.04 mmol), NHS (5 mg, 0.04 mol), and DMF (0.4 ml) was shaken at rt for 0.5 h. Then the resin was washed with DMF, and PBS quickly. After that 1/2SLPI(120 mg, 0.02 mmol) was added to the resin as a PBS solution (0.8 ml), and shaken at 4 °C for 15 min. After filtration, the resin was washed with ice-cold PBS and a mixture of the resin and PBS (0.4 ml) containing 2-ethanolamine (30 mg, 0.4 mmol) was shaken at 4 °C for 30 min. Then reaction solution was removed and resin was washed five times with ice cold PBS to afford the objective resin bearing 1/2SLPI (**5a**).

The other resins bearing 1/2SLPI (**5b**–**e**) was prepared in the same manner.

5.5. Preparation of rat brain lysate

Preparation of tissue extracts of rat brain. Fresh rat brain was homogenized (1:10, wt/vol) in buffer A. The homogenate was centrifuged at 9500 rpm for 10 min. After supernatant was separated, it was centrifuged at 50,000 rpm for 30 min again. Obtained supernatant was used for lysate and kept at −80 °C before use.

5.6. Binding assay on affinity resins with rat brain lysate

The lysate as a crude tissue extracts was diluted with buffer A and total protein concentration was about 7 mg/ml. One milliliter of this lysate was shaken calmly with 10 μl of prepared affinity resin at 4 °C for 0.5 h to bind the nonspecific binding proteins or specific binding proteins. A typical lysate solution has a total volume of 1.0 ml, consisting of buffer A, and 0.5 ml of tissue extract. After incubation, the resins were precipitated by centrifugation in a microcentrifuge at 12,000 rpm for 1 min. The resins were washed three times with buffer A. The washed resins were suspended in 30 μl of SDS–PAGE sample buffer solution (Nacalai, sample buffer solution with 2-ME(2×) for SDS–PAGE, cat. 30566-22, including 4%(w/v)-SDS, 20%(v/v)-glycerol, 0.01%(w/v)-BB, 10%(v/v)-2-ME, 0.125 M Tris, pH 6.8), shaken at 25 °C for 10 min. The supernatant was subjected to SDS–PAGE followed by CBB staining.

5.7. Preparation of HL-60 cell lysate

HL-60 cells (1×10^7) were freeze-thawed in lysis buffer [20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 2 mM EDTA] with Complete Protase Inhibitors (Roche Diagnostics). This solution was centrifuged at 10,000 rpm for 10 min. The supernatant was used for cell lysate and kept at −80 °C before use.

5.8. Binding and competition assay on affinity resins (5a, 5e, and 6) using HL-60 cell lysate

Binding assay using HL-60 cell lysate on affinity resin (**5a**, **5e**, and **6**) was performed in a same manner as using rat brain lysate. In the case of competition assay, the protocol was almost the same except addition of 1/2SLPI (0.5 mg) to cell lysate previously. And resulting eluates from resins were subjected to SDS–PAGE, followed by silver staining.

5.9. Western blot analysis

The proteins, obtained in binding and competition assay on affinity resins immobilized 1/2SLPI, were applied to SDS–PAGE followed by electroblotting onto PVDF membrane using the Invitrogen Xcell II™ blot module. After blocking with skim milk (Wako Pure Chemical industries, Ltd Cat. TCH7451) at rt for 30 min, the membrane was incubated with a mouse anti-L-plastin monoclonal antibody (Lab Vision-NeoMarkers, cat. MS-1326-P1ABX) at rt for 1 h and washed three times with PBS. Then the membrane was incubated with HRP-conjugated anti-mouse IgG (Amersham Biosciences Corp. cat. NA931V) at rt for 1 h and washed again. Protein bands were visualized using chemiluminesence-enhancing reagent (ECL Plus, Amersham Biosciences Corp. cat. RPN2132).

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