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Short communication

# Further application of a two-step heparin affinity chromatography method using divalent cations as eluents: Purification and identification of membrane-bound heparin binding proteins from the mitochondrial fraction of HL-60 cells

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**Abstract**

Membrane proteins were obtained from the mitochondrial fraction of HL-60 cells by solubilization with octyl glucoside and bound to heparin-gels. Bound proteins were successively eluted with solutions containing increasing concentrations of $Mg^{2+}$ in the first and increasing concentrations of $Ca^{2+}$ in the second chromatography. After SDS-PAGE and subsequent N-terminal amino acid analysis of proteins on each band, 13 proteins were identified. Fifteen out of the 37 proteins analysed were modified at their N-termini. These results show that this two-step affinity chromatography method using divalent cations as eluents can be applied to a variety of membranes for the isolation of specific proteins.


*Keywords:* Heparin binding proteins; Membrane proteins; Affinity chromatography; Divalent cations

## 1. Introduction

In the previous study we showed that divalent cations are effective for the elution of specific membrane proteins by heparin affinity chromatography [1]. We developed a two-step heparin affinity chromatography method to purify and analyse membrane proteins from the microsomal fraction of human promyelocytic leukemia cells, HL-60. In the first chromatography, solubilized proteins bound to heparin-gels were eluted with increasing concentrations of $Mg^{2+}$ and each fraction was then subjected to a second round of affinity chromatography using $Ca^{2+}$ as the eluent. This simple method allowed us to purify and identify eight proteins found in the microsomal fraction of HL-60 cells.

Divalent cations are essential for specific cellular functions, cell integrity and membrane stability. The attachment of some types of cells to the substratum also requires divalent cations [2]. Except for the integrin family proteins [3] and cadherins [4], the molecular mechanisms for divalent cation-dependent events on membranes have not been well understood, mainly due to the lack of information for proteins involved in these functions.

Increasing concentrations of $Na^{+}$ are usually employed to elute bound proteins from heparin-gels. To our knowledge, the use of divalent cations as eluents in heparin affinity chromatography or ion exchange chromatography has not been reported to date. In the light of the various aspects of divalent cations in specific functions and structural stability of membranes, it was of great interest whether the method is applicable to other membrane preparations. For this purpose, we utilized this method to isolate membrane-bound heparin binding proteins (MHBP) from the mitochondrial fraction of HL-60 cells.

*Abbreviations:* SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; MHBP, membrane-bound heparin binding proteins

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## 2. Experimental

### 2.1. Material

Heparin-Sepharose CL-6B and sulfated cellulose gels were obtained from Amersham Pharmacia Biotech and Seikagaku Kogyo, Tokyo, Japan, respectively. Other reagents were of reagent grade.

### 2.2. Cell culture and cell fractionation

Culture and fractionation of HL-60 cells were performed as described previously [1]. Briefly, cells grown to confluency in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum were collected and disrupted using a Dounce homogenizer. P5 fractions sedimenting at $5 \times 10^3 \times g$ by centrifugation for 15 min were used as the starting material for analysis of mitochondrial proteins, since this fraction contained 91% activity of the mitochondrial enzyme, succinate-cytochrome *c* reductase [1]. Microsomal (P40) fractions were obtained by further centrifugation at $4 \times 10^4 \times g$ for 90 min.

### 2.3. Assay

Protein concentrations were determined with the Coomasie brilliant blue G250 dye reagent (Bio-Rad), according to the manufacturer's instructions.

### 2.4. Affinity chromatography and amino acid sequencing

The procedure for purification of proteins in the mitochondrial fraction by heparin affinity chromatography is essentially the same as that described for the purification of proteins in the microsomal fraction in the previous paper [1]. Briefly, combined membrane fractions were solubilized in 1% octyl β-D-glucoside containing 10 mM sodium phosphate, pH 7.4, and protease inhibitors. Membrane extracts were obtained by centrifugation at $4 \times 10^4 \times g$ for 90 min. The extracts were first applied to sulfated cellulose column to remove proteins which bind non-specifically to sulfate residues or polysaccharides. The non-adsorbed proteins were then applied to the first heparin-gel column. After washing the column with the solubilizing buffer containing 0.15 M NaCl, proteins were eluted successively with 5, 20, 100 mM $MgCl_2$ in 1% octyl glucoside–10 mM sodium phosphate, pH 7.4 and finally with 2 M NaCl in the same buffer. Each of the four fractions was dialysed, concentrated and applied to the second heparin-gels. The bound proteins were then eluted successively with 1, 5, 20 mM $CaCl_2$ in 1% octyl glucoside–10 mM Tris–HCl, pH 7.4. Proteins were finally eluted with 2 M NaCl in the same buffer. Each of the four fractions and non-adsorbed fraction were dialysed, concentrated and purified by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in bands were blotted electrically to a PVDF membrane and stained in Coomassie brilliant blue R250. Protein samples in each band were analysed for their N-terminal amino acids with a Procise™ 492 cLC sequencing system (Applied Biosystems, CA, USA). Phenylthiohydantoin–amino acids were identified with a HPLC system consisting of model 140D gradient pump and model 785A UV–vis detector (Applied Biosystems). Sequence homology was analysed using the on-line PubMed provided by the National Center for Biotechnology Information, NIH, Bethesda, MD, USA or using FASTA program of EMBL-European Bioinformatics Institute (EBI) website.

## 3. Results and discussion

### 3.1. Heparin binding proteins in the mitochondrial fraction

Fig. 1 shows that MHBP in mitochondrial fractions of HL-60 cells can also be purified by the two-step affinity

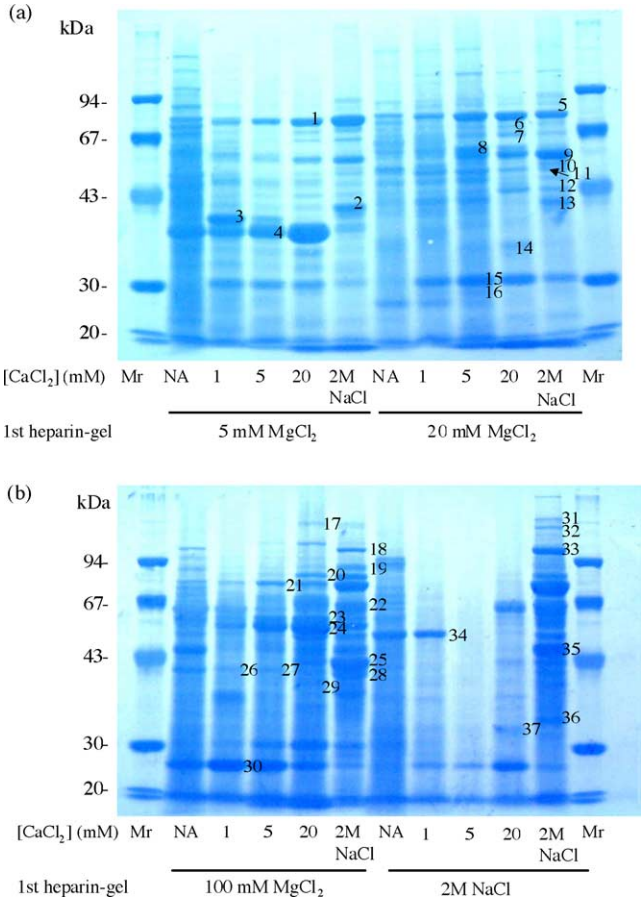


Fig. 1. Pooled P5 fractions were solubilized with 1% octyl glucoside and the extracted proteins (124 mg) were purified by two-step affinity chromatography with heparin-gels as described in Section 2.4. Fractions of 5, 20 (a), 100 mM $MgCl_2$ and 2 M NaCl (b) from the first heparin-gels were applied to the second heparin-gels. Proteins were eluted with 1, 5, 20 mM $CaCl_2$ and finally with 2 M NaCl. Each fraction after the second chromatography was finally purified by SDS-PAGE. Numbers on the bands indicate the protein bands analysed for their N-terminal sequences.

chromatography method [1]. Elution of proteins was dependent on ion concentration and ion species. From the electrophoretic patterns obtained after the second chromatography, the proteins can be classified into several groups. The first group contained proteins which were eluted with very low concentrations of $CaCl_2$, as low as 1 mM (bands 3 and 34). Proteins eluted with higher concentrations of $MgCl_2$ in the first chromatography were eluted with 1 mM $CaCl_2$ in the second chromatography. The capability of a low concentration of calcium ion to elute these proteins suggests that these proteins have a specific binding site with high affinity for calcium ions. The second group of proteins required high concentrations of divalent cations for their elution (bands 1, 4, 8, 9, 15 and 24). Both $MgCl_2$ and $CaCl_2$ were effective for the elution. The third group of proteins was eluted with increasing concentrations of $MgCl_2$, but was only eluted with 2 M NaCl in the second chromatography (bands 2, 10, 18, 19, 22, 25, 32, 33, 35 and 36).

### 3.2. Identification of MHBP in the mitochondrial fraction

Sequences of the 10 or more N-terminal amino acids of the proteins in the numbered bands were determined (Table 1). Membrane proteins identified included those from mitochondria (citrate synthase [5], hydroxyisobutyryl-CoA hydrolase [6], cytochrome $c_1$ [7], glutamate dehydrogenase [8]), endoplasmic reticulum (protein disulfide isomerase A6 [9], ERp29 [10], ERp57 (protein disulfide isomerase A3) [11], 150 kDa oxygen-regulated protein [12]), and plasma membranes ($Na^+$,$K^+$-adenosine triphosphatase [13]). A glycolytic enzyme, fructose 1,6-bisphosphate aldolase [14], and a nucleoplasmic enzyme, apurinic/apyrimidinic endonuclease [15], were associated with mitochondrial membranes as observed previously with glyceraldehyde 3-phosphate dehydrogenase in microsomes.

Since the present study was aimed to investigate effectiveness of the two-step heparin affinity chromatography method, a simple conventional cell fractionation was employed, which resulted in the cross contamination of organelles other than mitochondria in the P5 fractions. Recent organellar proteomics pioneered new sophisticated methods [16,17]. When combined with these methods, analysis by the present method may provide new information.

Since heparin modulates a variety of physiological processes, a common structural motif for heparin-binding in the interacting proteins has been searched and the consensus heparin-binding sequences (XBBXBX and XBBBXXBX) were identified [18], where B designates a basic amino acid and X other amino acid. We have searched these sequences within the MHBP of Table 1. Glutamate dehydrogenase and 3-hydroxyisobutyryl-CoA hydrolase contain QKRNRVR (97–103) and LHHLRM (18–23) corresponding to XBBXBX, respectively. Both XBBXBX and XBBBXXBX were found in oxygen-regulated protein: LRRVRQ (454–459) and AKHRFYRP (442–449). Though ERp29 and fructose 1,6-bisphosphate aldolase have been reported as heparin binding proteins [19,20], neither binding motif was present. Since several sequences other than the two above sequences have also been identified in heparin-binding region [21], MHBP of Table 1 may also contain other binding motifs.

N-terminal sequences of a considerable number of proteins could not be identified, presumably due to N-terminal modification (bands 1, 6, 7, 8, 16, 17, 19, 20, 21, 23, 26, 27, 33, 34 and 36). Information on the N-terminal modification of proteins has only been reported for cytoplasmic proteins of mammalian cells and bacteria [22], which revealed that more than 50% of proteins are modified. To date, such a study regarding N-terminal modification of membrane proteins has not been reported. The results in the previous and the present studies show that membrane proteins may also undergo the same incidence of modification. Other bands analysed showed the presence of multiple proteins in the bands.

Though two-dimensional PAGE has been reported to be difficult to apply for membrane proteins [23], detergent and buffer system were recently improved to separate membrane proteins [24]. However, sample preparation and buffer system for two-dimensional PAGE are still not satisfactory [25], since membranes contain proteins with wide ranges of p*I*,

Table 1
Sequences of the N-terminal amino acids of proteins from the mitochondrial fractions of HL-60 cells

| Band | Mr (kDa) | Amino acid sequence | Protein | Ref. no. |
|---|---|---|---|---|
| 2 | 38 | PYQYPALTPE | Fructose 1,6-bisphosphate aldolase | [14] |
| 3 | 37 | KHTDAXEEVL | 3-Hydroxyisobutyryl-CoA hydrolase | [6] |
| 4 | 35 | GKVKVGVNGF | Glyceraldehyde 3-phosphate dehydrogenase | [27] |
| 9 | 57 | SDVLELTDDN | ERp57 | [11] |
| 10 | 56 | SEAVADREDDP | Glutamate dehydrogenase | [8] |
| 11 | 53 | LYSSXDDNIEL | Protein disulfide isomerase A6 | [9] |
| 14 | 32 | TAAKKNDKEA | Apurinic/apyrimidic endonuclease | [15] |
| 15 | 28 | LHTKGALPLD | ERp29 | [10] |
| 25 | 12 | ASASSTNLID | Citrate synthase | [5] |
| 29 | 40 | TKNEKKXLNQ | $Na^+$,$K^+$-adenosine triphosphatase β-3 chain | [13] |
| 30 | 26 | ATGPRQYDGI | NipSnap3A | [28] |
| 32 | 103 | LAVMSVDLGDE | 150 kDa oxygen-regulated protein | [12] |
| 37 | 30 | SDLELHPPSYL | Cytochrome $c_1$ | [7] |

molecular weight and hydrophobicity. For this reason, SDS-PAGE was combined with a liquid chromatography–tandem mass spectrometry to identify mitochondrial proteins from yeast, [26].

Most of the proteins which were eluted with divalent cations and successfully identified have not been described to require divalent cations for their activity, except protein disulfide isomerase and endonuclease. Studies to re-evaluate the divalent cation requirement of these proteins may reveal new functions for divalent cations.

## 4. Conclusion

Proteins in the mitochondrial fraction of HL-60 cells were purified and analysed by the two-step affinity chromatography method, which made use of the divalent cations $Mg^{2+}$ and $Ca^{2+}$ as eluents for the heparin affinity chromatography. Thirteen MHBP were identified by N-terminal amino acid sequencing after SDS-PAGE. We suggest that this two-step affinity chromatography method using divalent cations as eluents is a convenient method which can also be applied to other membrane preparations. The method to isolate specific proteins should help progress organellar proteomics.

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