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Purification and liposomal reconstitution of the oligopeptide transport activity in rat renal cortex using ceftibuten-affinity chromatography

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Abstract

The carrier protein(s) responsible for the transport of ceftibuten, a peptide-like dianionic cefem, in rat renal brush-border membrane were solubilized and purified by a ceftibuten-ligand specific affinity chromatography technique. The proteoliposomes reconstituted from the solubilized brush-border membrane proteins by dialysis had H^+-sensitive uptake of ceftibuten and trans-stimulative effect by cephalexin. A specific uptake activity for ceftibuten was found in the 3.5 M-eluted fraction but not the flowthrough and the 0.5 M-eluted fraction of the affinity chromatography. Analyzing this active fraction by SDS/PAGE after reconstituting into liposomes gave two major proteins (approx. molecular masses of 130 and 107 kDa). The purification protocol presented in this study permitted an efficient isolation of the carrier proteins responsible for the transport of ceftibuten and other peptide-like compounds. © 1998 Elsevier Science B.V.

Keywords: Brush-border membrane; Ceftibuten; Oligopeptide transporter; Affinity chromatography; Liposomal reconstitution; Renal cortex; Rat

1. Introduction

Mammalian small intestine and kidney express transport systems which recognize substrates of endogenous origin such as glucose [1], amino acid [2], and oligopeptides [3–5]. Molecular cloning studies have revealed that the H^+-coupling oligopeptide transporters expressed in the intestine and kidney are structurally different, encoded by different genes [6–10]. The cloned intestinal peptide transporter (PepT1) and the kidney transporter (PepT2) of human and rat exhibited only approximate 50% homology in amino acid sequence. PepT2 consists of 729 amino acids and is expressed in the kidney and other tissues such as the brain, lung, liver and heart but not in the intestine, whereas PepT1 is expressed primarily in the intestine and, to much lesser extent, in the kidney. The studies made thus far on the transport machinery of oligopeptides, in spite of being extensive, still fall short in answering many questions such as: the absolute necessity of the proton gradient as a driving force; the contradictory results of the gene expression with those of the physicochemical approaches (radiation inactivation [11], purification [12] and photoaffinity labeling [13]) aimed at determining the molecular mass of the transporters; and last but not least the mechanism by which the transporters are regulated. On the basis of this introduction, functional purification of the carriers of interest would be the eventual way for unveiling these obscures. Our

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previous studies by kinetic analysis have indicated that multiple peptide transport systems are present in the renal brush-border membrane [14,15], and moreover, we could reconstitute the solubilized activity of ceftibuten transport in the brush-border membrane into liposomal preparations [16].

The present study describes an optimal protocol for the purification of the oligopeptide carrier proteins from rat renal cortex with the use of affinity chromatography on the immobilized substrate, ceftibuten.

2. Materials and methods

2.1. Materials

Ceftibuten, cephalexin and compound V, 3-vinyl derivative of ceftibuten [17] (Fig. 1) were kindly donated by Shionogi (Osaka, Japan). Octyl glucoside (*n*-octyl-β-D-glucoside) was purchased from Dojindo (Kumamoto, Japan). Asolectin (L-α-lecithin, commercial grade type II-S, Sigma, St Louis, USA) was cleaned up by the method of Sone et al. [18] beforehand. *p*-Trifluoromethoxy penylhydrazone (FCCP) were obtained from Sigma (St. Louis, Mo, USA). *N*-hydroxysuccinamide (NHS) activated sepharose column with a spacer arm of six carbon atoms between *N*-hydroxysuccinamide group and the matrix was purchased from Pharmacia (HiTrap NHS-activated, 5 ml, Uppsala, Sweden). All other chemicals were of reagent grade.

2.2. Isolation of brush-border membrane and solubilization of membrane proteins

The brush-border membrane (BBM) was isolated from the renal cortex of the male Wistar rat (200–

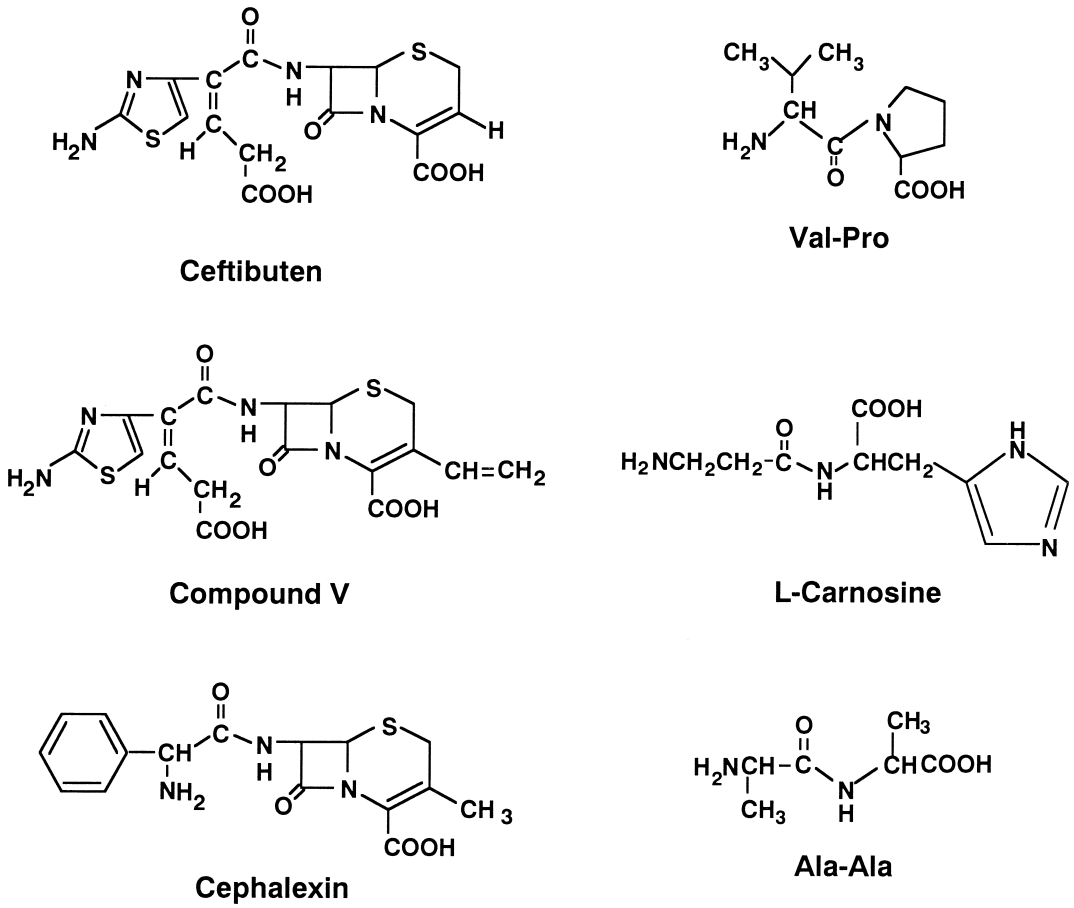

Fig. 1. The chemical structures of tested compounds.

260 g) by the Mg^{2+}/EGTA precipitation method of Biber et al. [19] with several modifications as in our previous paper [15]. The final pellet was resuspended in a buffer composed of 100 mM D-mannitol, 100 mM KCl and 20 mM Hepes–Tris (pH 7.5). The protein concentration was adjusted to 20 mg/ml, and the suspension was stored at −80°C under the nitrogen gas until use. The proteins from renal BBM were solubilized by suspending 30–40 mg of membrane proteins in 3 ml of solubilization buffer containing 60 mM octyl glucoside, 20 mM Hepes–Tris (pH 7.5), 100 mM D-mannitol, 100 mM KCl, 10% glycerol, 2 mM EDTA, 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After incubation on ice for 30 min with slow stirring, the supernatant was obtained from the centrifugation at $100\,000 \times g$ for 45 min, and used for the liposomal reconstitution.

Each protein fraction from either the initial solubilization or the affinity chromatography was mixed with asolectin micellar solution in the solubilization buffer (protein:lipid = 1:20, w/w), and readjusted the concentration of octyl glucoside to 50 mM. Liposomal reconstitution was achieved by dialysis against a 20 mM Hepes–Tris buffer (pH 7.5) containing 0.1 mM EDTA, 2.5 mM $MgCl_2$ and 0.1 mM DTT for 36 h at 4°C. The proteoliposomes were harvested by centrifuging the suspension at $100\,000 \times g$ for 1 h. The resulted pellet of proteoliposomes was suspended in a minimal volume of the experimental buffer for uptake measurement.

2.3. Immobilization of ceftibuten to the NHS-activated column

Ceftibuten, the selected ligand, was coupled to the activated gel according to the instruction of the supplier with minor modifications. Briefly, after washing the activated gel with three column volumes of 1 mM HCl at 4°C, 5 ml of a 50 mM ceftibuten solution in a carbonate buffer (pH 8.3) was injected into the column. The reaction was run for 2–3 h at room temperature. Then the remaining uncoupled NHS residues were inactivated by incubating with monoethanolamine. Finally, the column was washed three times with three column volumes of the carbonate buffer and of a 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, alternatively. The column was then washed with a phosphate buffer (pH 7.0) containing NaN_3 and stored at 4°C until use. The coupling ratio was found to be approx. 40% as estimated from the amount of ceftibuten remained in the washing solution.

2.4. Application onto the ceftibuten-affinity column

The solubilized brush-border membrane protein solution was 10-fold diluted with 20 mM Mes–NaOH buffer (pH 6.0) containing 50 mM NaCl, 1 mM EDTA, 0.3 mM DTT and 5 mM lactic acid in order to bring the detergent concentration below its critical micellar point, and applied onto the affinity column which was equilibrated in advance with the starting buffer. The starting buffer had the same composition of the buffer for dilution, but with the addition of 6.8 mM octyl glucoside. Protein elution was continuously monitored by UV absorbance at 280 nm (A_{280}). Following the loading of the membrane proteins, the column was washed with the starting buffer. When the A_{280} of the eluate reached the value of the starting buffer, the ionic strength of the eluent was step-wisely increased with NaCl. At first, 0.5 M NaCl removed the weakly bound proteins from the affinity column, and secondly, the highly bound proteins were eluted at 3.5 M NaCl. The fractions corresponding to the eluted peaks were collected in ice-cold tubes and processed for reconstitution, aminopeptidase analysis and protein estimation.

2.5. SDS-polyacrylamide gel electrophoresis

SDS/PAGE analysis was performed with an SPG-1500 Advantec vertical electrophoresis apparatus and the Laemmli [20] buffer system as described in the previous report [16]. A precast discontinuous slab gel ($1 \times 140 \times 140$ mm) containing 10% (w/v) acrylamide and 0.3% (w/v) N,N′-methylenebisacrylamide was obtained from Funakoshi (Funakoshi, EASY-GEL, Tokyo, Japan). Prior to SDS/PAGE the proteins from different membrane preparations were precipitated by the method of Wessel and Flügge [21] as modified by Kramer et al. [12]. The dried protein precipitates were dissolved in 40–100 μl of a buffer containing 50 mM Tris–HCl (pH 8.5), 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.002% (w/v) bromophenol blue. The mix-

Table 1
Purification of the ceftibuten transport activity from rat renal cortex

Step	Specific activity (pmol/mg protein/sec)	Purity (fold)	Yield (%)
Reversed phase evaporation technique			
Brush-border membrane	12.5	1.0	100.0
n-octyl glucoside extract	4.8	0.38	47.6
3.5 M-eluted fraction on affinity chromatography	15.8	12.7	0.47
Dialysis method			
Brush-border membrane	12.5	1.0	100.0
n-octyl glucoside extract	132.6	10.6	48.3
3.5 M-eluted fraction on affinity chromatography	3376.4	270.1	0.42

An aliquot at each step was reconstituted into proteoliposomes and the activity of ceftibuten transport in the presence of an inward H^+ gradient was determined. Final concentration of ceftibuten was 1.0 mM. Each data was expressed by the mean of three or four measurements.

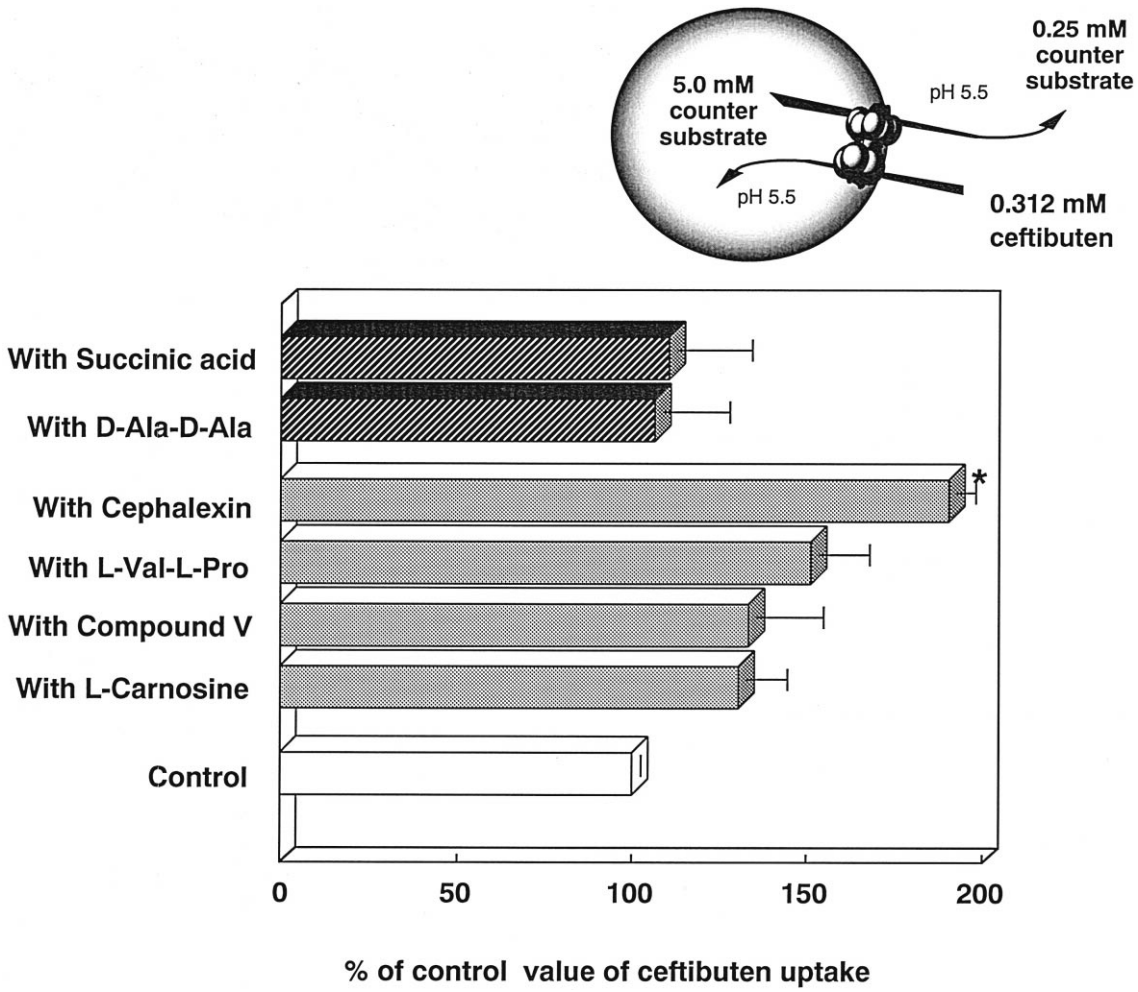

Fig. 2. Trans-stimulation effects of peptide-related compounds on the uptake of ceftibuten at early time period (5 min) by proteoliposomes reconstituted from the octyl glucoside crude extract. Vesicles (25 μl) were preloaded with counter-substrate (5 mM) at 30°C for 1 h. Uptake study was started by incubating the preloaded vesicles with 500 μl of ceftibuten solution (0.312 mM) in the absence of H^+-gradient ($pH_{in} = pH_{out} = 5.5$). Each value is the mean with SEM ($n = 4 \sim 5$).

ture was vortexed vigorously and incubated in a boiling water bath for 5 min. After the separation of protein at 25 mA, the gel was visualized with Coomassie-brilliant blue. Molecular-mass determinations were made by using a Sigma Calibration Unit (SDS-6H Sigma, St Louis, Mo, USA).

2.6. Uptake experiments

The uptake of ceftibuten into the prepared proteoliposomes was performed at 30°C by the rapid filtration method as described previously [16]. Unless specified other wise, uptake was initiated by mixing 40 μl of proteoliposomes with 200 μl of transport buffer (100 mM D-mannitol, 100 mM KCl and 20 mM Mes–Tris, pH 5.5) containing substrates at 30°C. The reaction was stopped with the addition of 4 ml of an ice-cold stop solution containing 150 mM NaCl and 20 mM Hepes–Tris, pH 7.5. The mixture was rapidly filtered through a Millipore filter (HAWP, 0.45 mm, 2.5 cm diam.). The filter was then washed 4 ml of the ice-cold stop solution. Ceftibuten trapped on the filter was extracted with 300 μl of the stop solution, and then analyzed by HPLC.

2.7. Analytical procedures

The detection of ceftibuten was carried out by HPLC as described previously [16]. Protein was measured by the method of Lowry et al. [22] with bovine serum albumin as the standard. Lipid phosphorus was measured by the ashing technique of Bartlett [23]. Aminopeptidase activity was assessed in the eluted fractions using the Wako® assay kit (LAP IC, Osaka, Japan). After removing any traces of the peptidase inhibitor, EDTA by ultrafiltration using a 10 000 kDa cutoff ultrafilter (Millipore, UFP2, LGC24, Tokyo, Japan), the resulted aliquot was used for assay of peptidase activity either directly or after reconstituting into liposomes according to the routine procedures of reconstitution mentioned above.

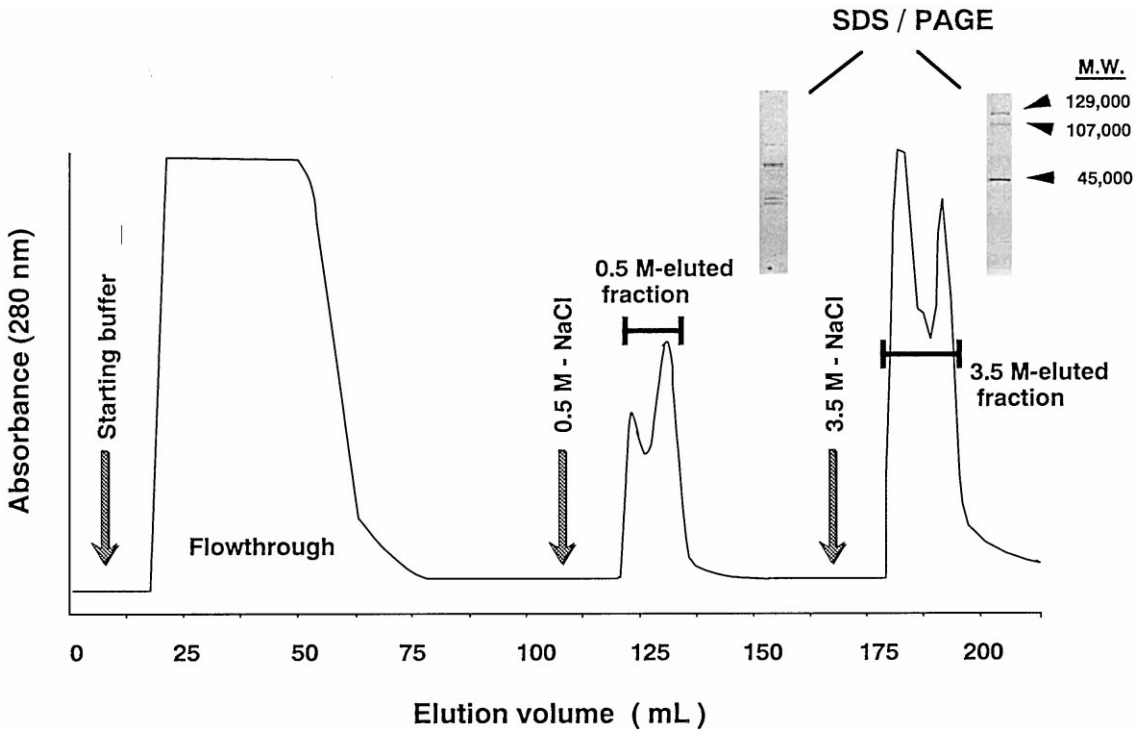

Fig. 3. Affinity chromatography of the *n*-octyl glucoside extract from the renal brush-border membrane for the purification of ceftibuten transport activity. Solubilized proteins (octyl glucoside extract) were fractionated on a ceftibuten-affinity column as described under Section 2. SDS/PAGE analysis of the eluted fractions (0.5 M and 3.5 M NaCl-eluted fraction) were shown in the insets, respectively.

3. Results

3.1. Reconstitution of H^+-cotransport activity into the asolectin liposomes

Liposomal preparation by dialysis had an initial uptake rate of 132.6 pmol ceftibuten/mg protein per second. These values are approximately 25-fold greater than those obtained from the freeze/thaw technique. (Table 1). To confirm the viability of the carrier protein(s) responsible for ceftibuten transport, trans-stimulation study was made as shown in Fig. 2. In the presence of D-cephalexin and L-valyl-L-proline inside the vesicles, the uptake was increased 200% and 150%, respectively. Moreover, compound V and L-carnosine also enhanced the uptake of ceftibuten although its stimulative effects were not significantly different from control value. This result extends the evidence of the functionality of the reconstituted carrier protein(s) in the present method.

3.2. Purification of the transport activity by affinity chromatography

Fig. 3 shows the elution profile after applying the solubilized membrane proteins onto the affinity matrix containing ceftibuten coupled via its free amino group. Uptake experiments on proteoliposomes reconstituted from the flowthrough and the 0.5 M NaCl-eluted fractions revealed that these fractions did

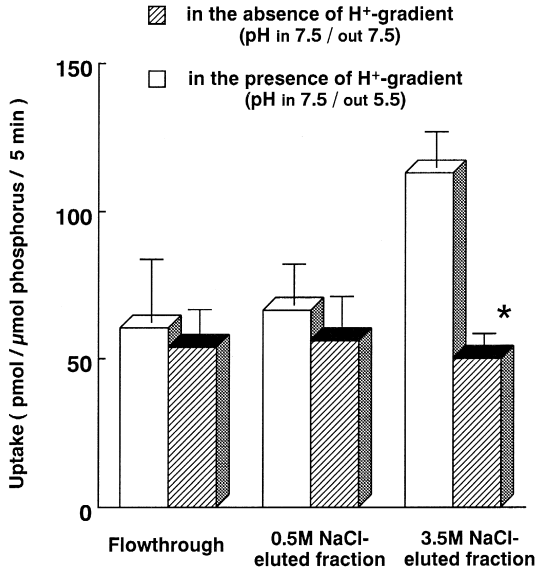

Fig. 4. Uptake activity of ceftibuten by proteoliposomes prepared by the various fractions after eluting from the affinity column. Uptake study was perform in the presence or absence of an inward H^+-gradient. Each value represents the mean with SEM ($n = 5$). $^*P < 0.05$ significantly different from uptake value in the presence of an inward H^+-gradient (opened column).

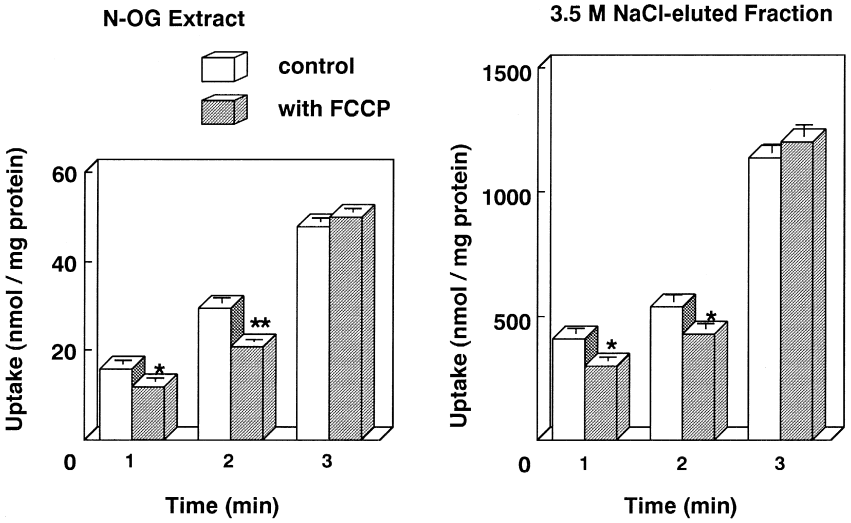

Fig. 5. Uptake of ceftibuten (1.0 mM) by proteoliposomes reconstituted from the octyl glucoside crude extract or the 3.5 M NaCl-eluted fraction of the affinity chromatography. Uptake experiments were performed in the presence of an inward H^+ gradient ($pH_{in} = 7.5$, $pH_{out} = 5.5$) with (hatched column)/without (opened column) FCCP (50 μM). Each value represents the mean with SEM ($n = 4$). $^{**}P < 0.01$, $^*P < 0.05$ significantly different from control (opened column).

not seem to have a specific uptake activity for ceftibuten. In contrast, the liposomal reconstitution of the 3.5 M NaCl-eluted fraction showed a higher uptake activity in the presence of an inward H^+-gradient (Fig. 4). This uptake activity was able to be significantly inhibited by the presence of FCCP, a protonophore, as shown in Fig. 5. Since there is a possibility that in addition to the oligopeptide transporters, the aminopeptidase which is a membrane enzyme, might also be able to recognize ceftibuten as a ligand, the activity of this enzyme was determined before and after liposomal reconstitution and in the absence of EDTA. As a result, no activities could be found in both of the reconstituted and the free proteins (not shown), indicating that the 3.5 M NaCl-eluted proteins do not contain aminopeptidase.

3.3. SDS / PAGE analysis

Analyzing the 3.5 M-eluted fraction on the affinity chromatography by SDS/PAGE before the liposomal reconstitution gave three proteins with apparent molecular masses of 129 ± 5, 107 ± 5 and 45 ± 1 kDa (Fig. 3). However, analyzing the same fraction by SDS/PAGE, after reconstituting into liposomes, yielded only the former two proteins, indicating an inability of the 45 kDa protein to be reconstituted into the bilayer of the liposomal membrane (Fig. 6).

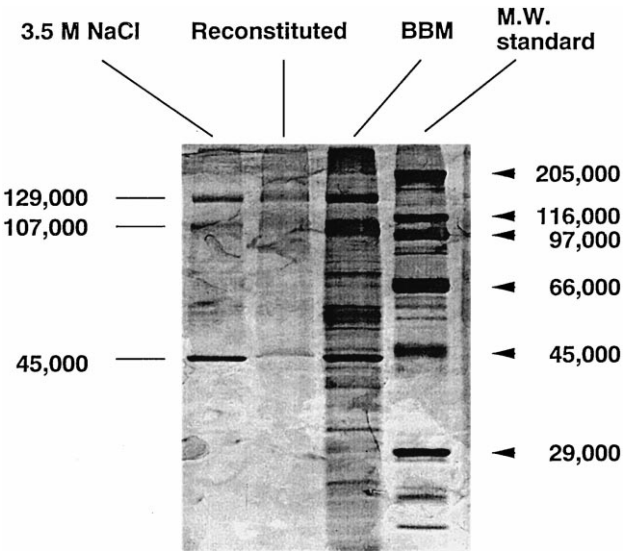

Fig. 6. SDS/PAGE analysis of the highly bound (3.5 M NaCl-eluted) fraction from the affinity chromatography before and after reconstitution into liposomes at a lipid-to-protein ratio of 20. BBM; Brush-border membranes, Reconstituted; proteoliposomes prepared by 3.5 M-eluted fraction, 3.5 M NaCl; 3.5 M NaCl-eluted fraction before reconstitution.

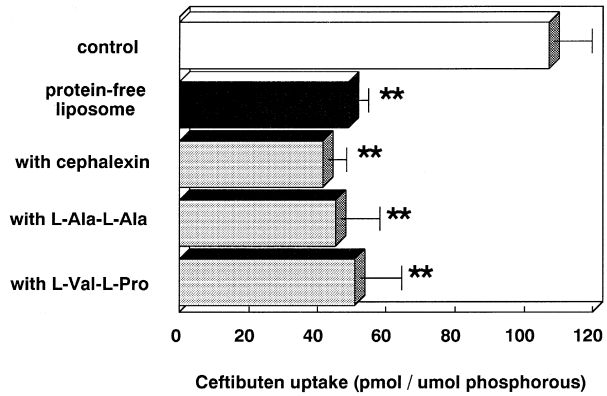

Fig. 7. Inhibitory effects of dipeptides and cephalexin on the uptake of ceftibuten (0.5 mM) by proteoliposomes prepared from the 3.5 M NaCl-eluted fraction of affinity chromatography. Uptake was measured in the presence of an inward H^+-gradient ($pH_{in} = 7.5$, $pH_{out} = 5.5$). Protein-free liposomes were prepared by the same dialysis method without protein fraction. Inhibitors were added at a concentration of 10 mM. Each value is the mean with SEM ($n = 8 \sim 10$). $^{**}P < 0.01$, significantly different from control.

3.4. Substrate specificity of the proteoliposomes reconstituted from the 3.5 M-eluted fraction of the affinity chromatography

The inhibitory effects of dipeptides and cephalexin were examined in the presence of an inwardly directed proton gradient. As shown in Fig. 7, the liposomal preparation of this study was characterized with an inhibition behavior which is similar to that observed in the native renal brush-border membrane vesicles [14,15] and in the primary proteoliposomes reconstituted from the total membrane protein solubilized [16]. Furthermore, the uptake value of ceftibuten by the protein-free liposomes was limited and very close to the uptake values in the presence of inhibitors, confirming the functionality of the reconstituted carrier protein(s) in this preparation.

4. Discussion

In our previous study [16], we have demonstrated a freeze/thaw technique for the reconstitution of the carrier system(s) responsible for the transport of ceftibuten and other peptide-related compounds in the renal brush-border membrane. In the present study, proteoliposomes were prepared by dialysis method, and was compared with those prepared by the previous method [16]. Finally, proteoliposome reconstituted by dialysis from 3.5 M-eluted fraction on affinity chromatography had an initial uptake rate of 3.38 nmol ceftibuten/mg protein per second. These values are approximately 21-fold greater than those obtained from the freeze/thaw technique. (Table 1). Most of the transport characteristics observed in the native brush-border membrane vesicles [14,15] were also observed in the reconstituted preparation of the present study.

Kramer et al. [13] have mentioned that their attempts to purify the oligopeptide transporter from the intestinal brush-border membrane with cephalexin specific affinity chromatography had a slight success, resulting in a tiny amount ($< 1\,\mu g$) of the putative carrier protein (130 kDa). The success of the protocol presented in this study could be explained by the following remarks: (1) in contrast to the protocol of Kramer et al., the detergent concentration of the membrane preparation was brought down, before application onto the column, to a level below the critical micellar concentration, permitting the carrier proteins to restore their functional conformation; (2) in addition to the fact that ceftibuten has a stronger affinity to oligopeptide transporter than cephalexin, ceftibuten was coupled to the affinity matrix via its free amino group which is not a part of the oligopeptide backbone; whereas, this is not the case for cephalexin which has been linked via its α-amino group; (3) the addition of exogenous lipids to the solubilized membrane preparation is thought also to assist in restoring the active form of the transporters.

In the renal brush-border membrane, the presence of two proteins with the ability to recognize oligopeptides has been already assessed by kinetic analysis [15], gene expression [24] and photoaffinity labeling and target size (radiation inactivation) techniques [11] using cefadroxil as a substrate. The molecular masses of these protein determined by target size analysis (105 and 130 kDa) are in good agreement with the proteins isolated in the present study. However, the 130-kDa protein is appeared to be a brush-border binding protein for cephems such as cefadroxil and ceftibuten because that this protein was photoaffinity-labeled by [^{3}H] cefadroxil even in the absence of any driving force as described by Boll et al. [11].

Renal brush-border membrane vesicles for peptide transport studies are normally prepared from the entire portion of the cortical tissue, which contains not only the proximal convoluted tubule but also the distal parts of the proximal tubule. Therefore, our previous results [15] showed that the high-affinity/low-capacity as well as the low-affinity/high-capacity peptide transport systems were detectable in these membrane vesicles. Brandsch et al. [25] demonstrated the presence of the high-affinity system PepT2 and of the low-affinity system PepT1 in the proximal tubular epithelial cells of the normal kidney. Additionally, Boll et al. [24] isolated a single clone (PepT2), expressing the peptide transport activity, from cDNA library of rabbit renal cortex, and reported that in vitro translation of this gene using a rabbit reticulocyte lysate with canine pancreatic microsomes yielded translation product with molecular mass of 107 kDa (core protein molecular size of 83 kDa).

In the present study, the result of affinity chromatography showed the proteins with apparent molecular size of 130- and 107-kDa had affinity for ceftibuten-ligand. It is speculated that larger protein is the binding protein as mentioned by Boll et al. [11], and that smaller one is associated with PepT2 transporter although it will be necessary to isolate and confirm the transport activity of each protein in the liposomal reconstitution.

On the other hand, the oligopeptide transport system in the renal brush-border membrane were reported to be under the regulation of protein kinase C as in the intestinal transport system [26]. Therefore, the 45 kDa polypeptide found in the highly bound fraction of affinity chromatography may be a component of protein kinase C associated to the carrier proteins during the purification process, although the possibility that it is the actin derived from the membrane filament was not excluded.

In conclusion, the purification protocol presented

in this study permitted an efficient isolation of the carrier proteins responsible for the transport of ceftibuten and other peptide-like compounds. The classification and the physicochemical properties (amino acid sequence, glycosylation, dimensional structure, etc.) of the isolated protein will be the subject of further studies.

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