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Purification by ceftibuten-affinity chromatography and the functional reconstitution of oligopeptide transporter(s) in rat intestinal brush-border membrane

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Abstract

The transport activity of ceftibuten, a dianionic peptide-like compound, was extracted from rat intestinal brush-border membrane by *n*-octylglucoside and reconstituted into asolectin liposomes by dialysis. The proteoliposomes prepared from the membrane extract showed an inward H^+-gradient-dependent uptake of ceftibuten and glycylsarcosine. Ceftibuten-immobilized affinity chromatography of the membrane extract permitted the isolation of two polypeptides (apparent molecular mass of 117 and 127 kDa) that can recognize the dianionic peptide structure of ceftibuten. Proteoliposomes prepared from reconstituting the isolated proteins into asolectin vesicles showed an overshooting uptake of ceftibuten in the presence of an inwardly directed H^+ gradient, and this uptake could be inhibited by L-valyl-L-proline. *N*-glycanase digestion of the isolated proteins, 117 and 127 kDa, trimmed them into 78 and 120 kDa products, respectively. The protein core size of the smaller protein was in agreement with the calculated molecular mass of ~79 kDa for the rat PepT1 transporter obtained by other investigators. © 1998 Elsevier Science B.V.

Keywords: Oligopeptide transporter; Ceftibuten; Glycylsarcosine; Affinity chromatography; Reconstitution; Brush-border membrane; Small intestine

1. Introduction

Oligopeptide transporters, which occupy a promising rank for pharmaceutical purposes, are specific for di-, and tri-peptide (but not amino acids) and peptide-like compounds such as β-lactam antibiotics, angiotensin-converting enzyme inhibitors, renin inhibitors and bestatin [1–4]. From a physiological viewpoint, the renal and small intestinal peptide transport systems play a vital role in the maintenance of nitrogen balance and protein nutrition, whereas from a pharmacological viewpoint, the low specificity and the ability of these transporters to interact with a large number of structures are attractive features that help in the rational design of peptidomimetic drugs with improved pharmacodynamic properties.

The use of various techniques such as isolated membrane vesicles [5,6] and intestinal cell culture [7] have contributed greatly to the characterization of the

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oligopeptide transport system in the intestinal epithelia. Recently, a cDNA encoding an oligopeptide transporter (PepT1) was isolated from the rat [8], rabbit [9] and human [10] small intestines, and the transport characteristics were assessed by gene-expression systems. Yet it is considered that the physical isolation of the carrier proteins is a prerequisite for a complete molecular description of the function of these transport systems. Since the main problem associated with the functional isolation of the carrier proteins stems from the instability of these proteins toward harsh conditions during the processing for purification, the determination of the proper conditions for the extraction and reconstitution into an environment approximating the native membrane is an integral and important part of the strategy for purification. Our previous papers, [11,12], described the proper conditions for the solubilization, purification and functional reconstitution of the system(s) responsible for the transport of ceftibuten, a peptide-like compound, in rat renal brush-border membrane. For the small intestine, however, only one method with a lengthy protocol was reported by Kramer et al. [13] for the purification of rabbit small intestinal oligopeptide transporter.

The purpose of the present study is to establish a protocol for the purification and the functional reconstitution of the oligopeptide carrier protein(s) from rat small intestine using affinity chromatography with the immobilized substrate, ceftibuten. As described in our previous studies [11,12], ceftibuten, a water-soluble dianionic cephem antibiotic, was used as a model substrate. The availability of a high performance liquid chromatography (HPLC) method for detecting ceftibuten at a relatively high level of sensitivity (~ 5 pmol), the ability of this compound to interact specifically with the peptide transporter [14,15] and the stability in aqueous media or against hydrolyzing enzymes (peptidase and β-lactamase), altogether, were reasons for the selection of ceftibuten as a model substrate.

2. Materials and methods

2.1. Materials

Ceftibuten and compound V, a 3-vinyl derivative, were kindly donated by Shionogi (Osaka, Japan). *n*-octylglucoside (*n*-octyl-β-D-glucoside) was purchased from Dojindo (Kumamoto, Japan). Asolectin (L-α-lecithin, commercial grade type II-S), dipeptides were from Sigma (St. Louis, Mo, USA), and asolectin was cleaned up by the method of Sone et al. [16] beforehand. *N*-Hydroxysuccinamide activated sepharose column with a spacer arm of six carbon atoms between the *N*-hydroxysuccinimide group and the matrix was purchased from Pharmacia (HiTrap NHS-activated 5 ml, Uppsala, Sweden). *N*-Glycanase (peptide-N^4(*N*-acetyl-β-glucosaminyl) asparagine amidase; EC 3.5.1.52) was obtained from Genzyme (Cambridge, MA). The enzyme was dialyzed against 0.2 M sodium phosphate buffer, pH 8.6, prior to use to remove glycerol. [^{14}C] Glycylsarcosine (4.07 GBq mmol^{-1}) was purchased from Moravek Biochemcals (Brea, CA, USA). All other chemicals were of reagent grade.

2.2. Solubilization of brush-border membrane proteins

The brush-border membrane (BBM) fraction was isolated from the small jejunum of the male Wistar rat by the divalent cation precipitation method of Kessler et al. [17] with several modifications as in our previous paper [14]. The final pellet was resuspended in a buffer composed of 100 mM mannitol, 100 mM KCl, and 20 mM Hepes-Tris (pH 7.5). The protein concentration was adjusted to 20 mg/ml, and the suspension stored at −80°C until use.

BBM proteins were solubilized with 50 mM *n*-octylglucoside at a protein concentration of 10 mg/ml in the presence of 1 mM DTT (dithiothrietol), 50 μM PMSF (phenylmethylsulfonyl fluoride) 10% glycerol, 3 mM EDTA, 100 mM KCl and 20 mM Hepes-Tris (pH 7.5). After centrifuging at $100\,000 \times g$ for 45 min, the clear supernatant was used for liposomal reconstitution and further purification.

2.3. Application onto the ceftibuten-affinity column

As illustrated in Fig. 1, ceftibuten was coupled to the NHS-activated gel according to the method described our previous study [12]. Transport activity was purified by applying the solubilized BBM proteins onto the ceftibuten-affinity column equilibrated with a starting buffer containing 50 mM NaCl, 1 mM EDTA, 0.3 mM DTT, 5 mM lactic acid, 6.8 mM *n*-

octylglucoside and 20 mM Mes-Tris (pH 6.0) as described in our previous report [12]. Following the decrease of the absorbance at 280 nm of the eluate to the basic level, the weakly bound proteins eluted from the affinity column by a 0.5 M NaCl in the starting buffer. Then 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, SDS/PAGE, and protein estimation.

2.4. Reconstitution into proteoliposomes

Each protein fraction from either the initial lysate or the affinity chromatography eluates was mixed with an asolectin micellar solution in 50 mM *n*-octylglucoside (protein : lipid = 1 : 20 w/w), and the mixture was dialyzed against 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 centrifugation at $100\,000 \times g$ for 1 h. The resulted pellet of proteoliposomes was resuspended in a minimal volume of the experimental buffer for uptake measurement.

2.5. Uptake experiments

The uptake of ceftibuten or [^{14}C]glycylsarcosine into the prepared proteoliposomes was performed at 30°C by the rapid filtration method as described previously [11,12]. The reaction was initiated by the mixing with an uptake buffer (100 mM mannitol, 100 mM KCl, 20 mM Mes-Tris, pH 5.5) containing substrates. After the stated time, the reaction was stopped by diluting the mixture with 4 ml of an ice-cold stop solution containing 150 mM NaCl and 20 mM Hepes-Tris, pH 7.5. The contents of the tube were immediately filtered under vacuum through a Millipore filter (HAWP, 0.45 mm, 2.5 cm diameter). The filter was then washed once with 4 mL of the ice-cold stop buffer. Substrates trapped on the filter were extracted with 300 μl of the stop buffer.

2.6. Analytical procedures

The detection of ceftibuten was carried out by the use of HPLC as described previously [12]. The concentration of [^{14}C] glycylsarcosine was determined by liquid scintillation counting. Protein was measured by the method of Lowry et al. [18] with bovine serum albumin as the standard. Lipid phosphorus was measured by the ashing technique of Bartlett [19].

2.7. SDS-polyacrylamide gel electrophoresis

SDS/PAGE analysis was performed using a commercially available minislab gel (MULTI GEL type-10(84(W) × 90(H) × 1 mm), 10% (w/v) acrylamide and 0.3% (w/v) *N*,*N*′-methylenebisacrylamide)

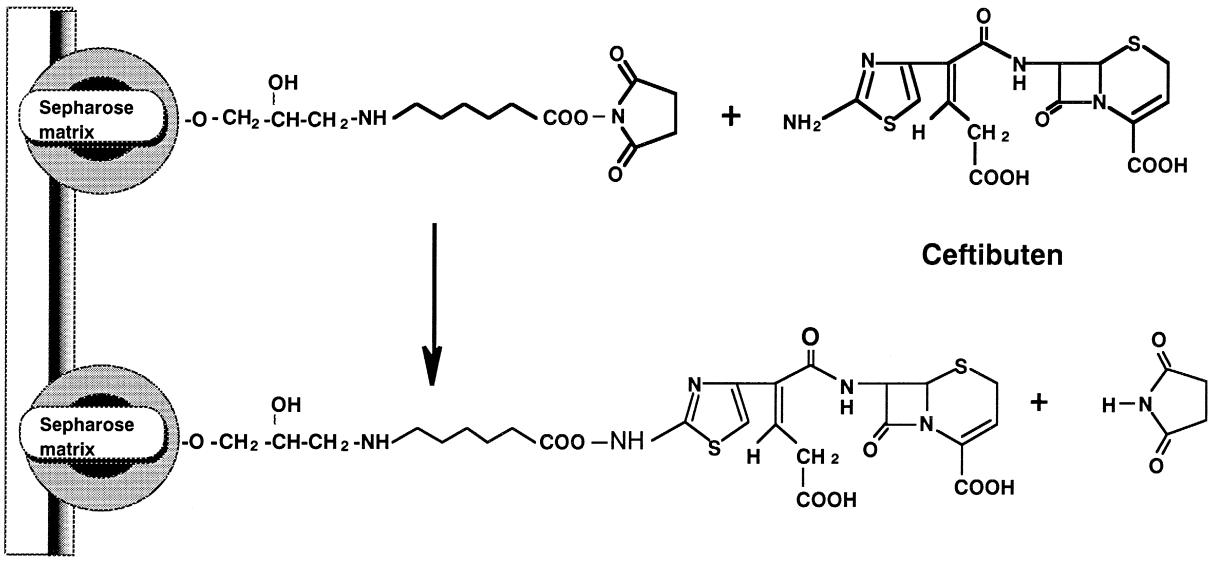

Fig. 1. Immobilization of ceftibuten to an activated *N*-hydroxysuccinimide (NHS) activated sepharose matrix.

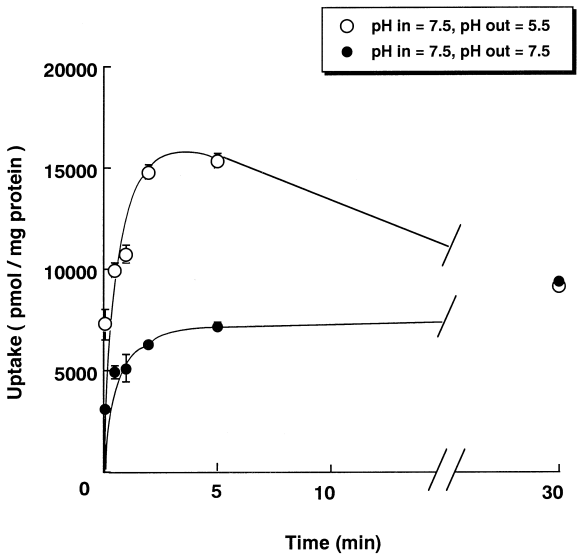

Fig. 2. Uptake profile of ceftibuten (1.0 mM) by proteoliposomes prepared from reconstituting the total protein extract of the intestinal brush-border membranes into asolectin lipid vesicles by dialysis method. Uptake was measured in the presence, or absence of an inward H^+ gradient ((○) – (pH_{in} 7.5)/(pH_{out} 5.5); (●) – $pH_{in} = pH_{out} = 5.5$). Each value is the mean ± SEM ($n = 4$).

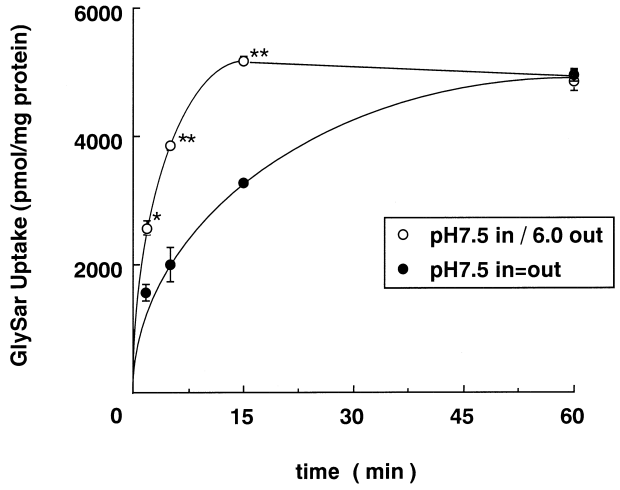

Fig. 4. H^+-gradient-dependent glycylsarcosine uptake by the proteoliposomes prepared from reconstituting the total protein extract of the intestinal brush-border membranes into asolectin lipid vesicles by dialysis method. Uptake was measured in the presence, or absence of an inward H^+ gradient ((○) – (pH_{in} 7.5)/(pH_{out} 5.5); (●) – $pH_{in} = pH_{out} = 5.5$). Each value is the mean ± SEM ($n = 6$). * $P < 0.05$; Significantly different from control, ** $P < 0.01$.

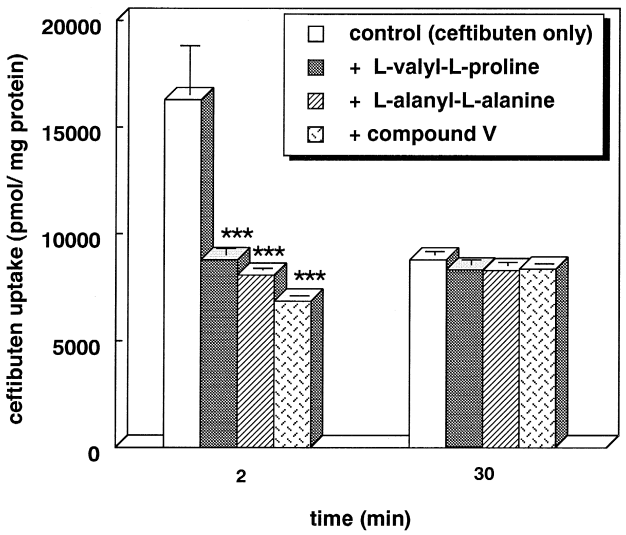

Fig. 3. Effect of Compound V and dipeptides on the initial (0.5 min) uptake of ceftibuten (1.0 mM) by the proteoliposomes prepared from reconstituting the total protein extract of the intestinal brush-border membranes into asolectin lipid vesicles by dialysis method. Uptake was measured in the presence of an inward H^+ gradient (pH_{in} 7.5, pH_{out} 5.5). Each value represents the mean ± SEM ($n = 4$). *** $P < 0.001$, Significantly different from control.

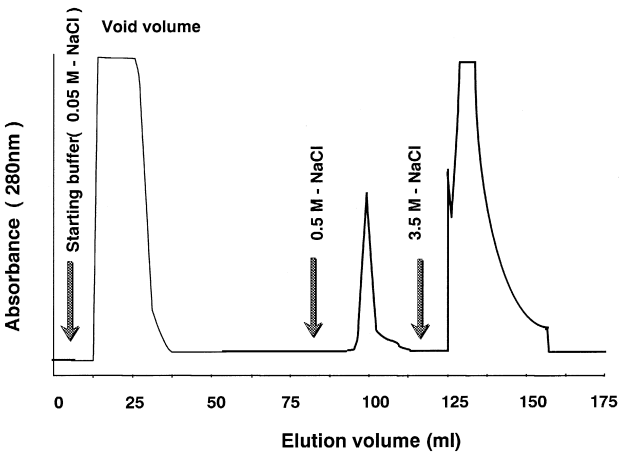

Fig. 5. Affinity chromatography of the *n*-octylglucoside extract of rat intestinal brush-border membrane for the purification of ceftibuten transport activity. Solubilized proteins (*n*-octylglucoside extract) were fractionated on a ceftibuten-immobilized affinity column as described under Section 2. Uptake activities of ceftibuten (1.0 mM) were determined using proteoliposomes prepared from the void volume, the 0.5 and 3.5 M NaCl-eluted fractions.

equipped with a Model DPE-220C vertical electrophoresis apparatus (Daiichi, Tokyo, Japan) and the Laemmli [20] buffer system. Prior to SDS/PAGE, the proteins from the different fractions were precipitated by the method of Wessel and Flügge [21] as modified by Kramer et al. [22]. The dried protein precipitates were dissolved in 40–50 μl of a loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. The mixture was vigorously vortexed for 2 min and then incubated for 5 min in a boiling water. Resolving the proteins at 40 mA using the electrode buffer (pH 8.4) containing 25 mM Tris, 192 mM glycine and 10% SDS, the gel was visualized with Coomassie brilliant blue staining. Molecular-mass determinations ware made by using a Sigma calibration unit (SDS-6H, Sigma). Deglycosylation of 3.5 M-NaCl eluted fraction by *N*-glycanase was according to the method of Hirai et al. [23] as described in the legend of Fig. 8.

3. Results

3.1. Solubilization and liposomal reconstitution of the H^+/ceftibuten co-transport activity

Our previous report [11] showed that an *n*-octyl-glucoside concentration of 50–60 mM is optimal for extracting the uptake activity of ceftibuten from the renal BBM. In the present study, proteoliposomes prepared from the total extract of the small intestinal BBM by dialysis had an initial uptake rate of 7.5 nmol ceftibuten/mg protein per min with an overshooting accumulation of 802.02 ± 19.94 pmol ceftibuten/μmol phosphorus (15.0 nmol/mg protein) at 2 min-incubation. These values are approximately three- and fourfold, respectively, greater than those obtained in the absence of an inward H^+ gradient (Fig. 2).

Fig. 3 shows that compound V, a ceftibuten analog, and dipeptides significantly inhibited the uptake

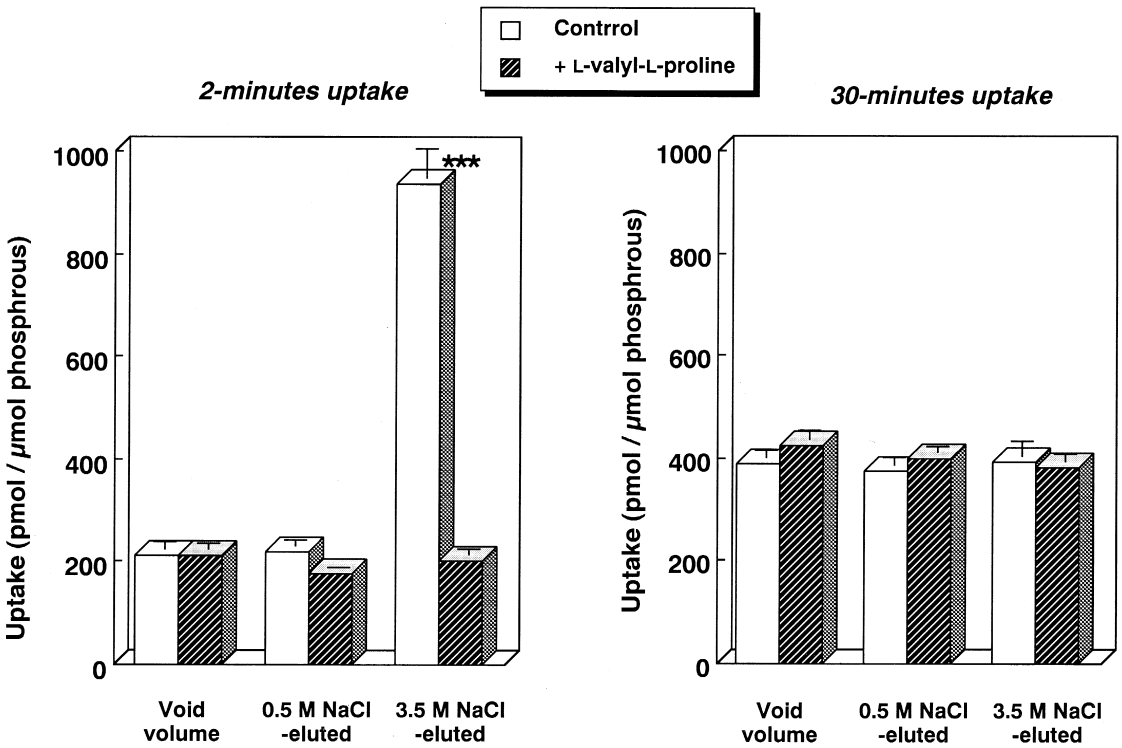

Fig. 6. Uptake of ceftibuten (1.0 mM) by proteoliposomes prepared from the void volume, the 0.5 or 3.5 M NaCl-eluted fraction of affinity chromatography. Uptake experiments were performed in the presence of an inward H^+ gradient ($pH_{in} = 7.5$, $pH_{out} = 5.5$) with (hatched column)/without (opened column) L-valyl-L-proline (10 mM). Each value represents the mean with SEM ($n = 4$). *** $P < 0.001$, Significantly different from control (opened column).

of ceftibuten into the proteoliposomes of the total membrane extract. Furthermore, the values of equilibrium uptake at 30-minute incubations were almost the same in the presence, or absence of inhibitors, indicating that the presence of inhibitors did not affect the vesicle size. Additionally, [^{3}H]-glycylsarcosine uptake into these proteoliposomes exhibited a distinct stimulation by an inwardly directed H^+ gradient (Fig. 4) which was similar to that observed in the original BBM vesicles (data not shown). The present results give an evidence that the transport activities of dipeptides in the proteoliposomes of the total BBM extract are similar to those described in the original brush-border membrane vesicles [24].

3.2. Ceftibuten-immobilized affinity chromatography of the intestinal BBM proteins

Fig. 5 illustrates the elution profile after applying the solubilized BBM proteins on the affinity column. As in the case of renal preparation [12], the liposomal reconstitution of the void volume and the 0.5 M NaCl-eluted fractions revealed that these fractions do not seem to have a specific uptake activity for ceftibuten, whereas the proteoliposomes reconstituted from the 3.5 M NaCl-eluted fraction showed a high uptake activity which was completely inhibited by the dipeptide, L-valyl-L-proline (Fig. 6). The equilibrium uptake values into all the three kinds of proteoliposomes were almost similar, indicating that the vesicles in these liposomal preparations are of similar size.

3.3. SDS / PAGE of the membrane protein fractions obtained from ceftibuten-immobilized affinity chromatography

Analyzing each purification step by SDS/PAGE revealed the presence of two polypeptides with apparent molecular masses of 127 ± 5 and 117 ± 5 in the 3.5 M NaCl-eluted fraction (Fig. 7). These polypeptides were not found in the 0.5 M NaCl-eluted fractions. Due to the fact that the SDS/PAGE analysis of the 40 mM ceftibuten-eluted fraction also showed a similar result to the 3.5 M NaCl-eluted fraction (data not shown), the highly bound proteins eluted by 3.5 M NaCl seems to be specific for

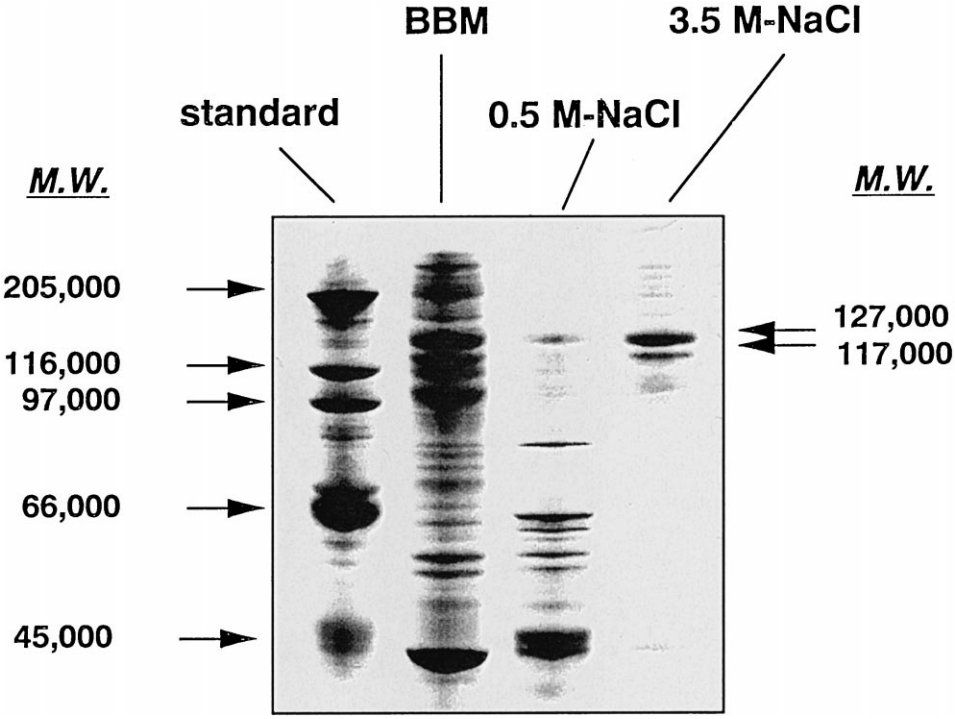

Fig. 7. SDS/PAGE analysis of the eluted fractions of affinity chromatography. BBM, crude extract of brush-border membrane proteins; 0.5 M-NaCl, 0.5 M NaCl-eluted fraction; 3.5 M-NaCl, 3.5 M NaCl-eluted fraction of ceftibuten-immobilized affinity chromatography.

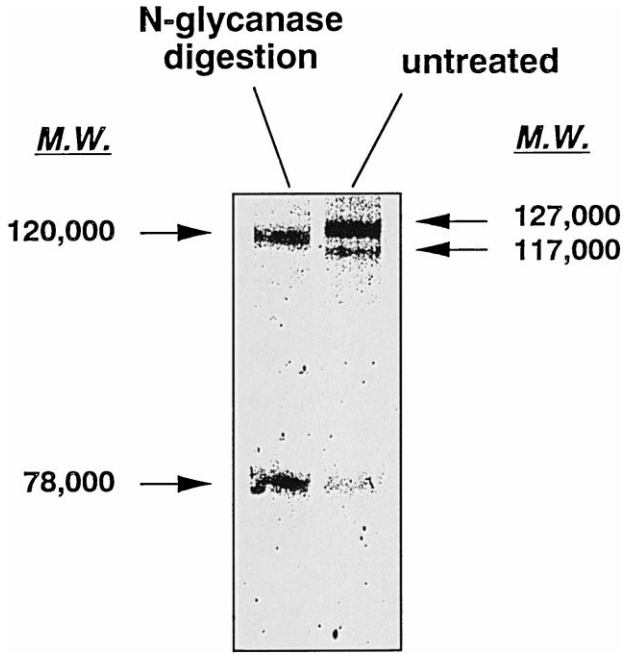

Fig. 8. Effect of deglycosylation by *N*-glycanase on the band-shift of 3.5 M-eluted fraction on SDS/PAGE analysis. After dialysis of 3.5 M NaCl-eluted fraction against 50 mM phosphate buffer (pH 8.6) containing 0.5% SDS and 50 mM β-mercaptoethanol, 5 μl of 7.5% Nonidet p-40 (NP-40) and 0.3 U of *N*-glycanase were added to 10 μl of protein sample aliquot, and the sufficient distilled water to a final volume of 30 μl was added. The mixture was incubated for 18 h at 37°C. The reaction was stopped by boiling for 5 min.

ceftibuten, but not due to a non-specific adsorption. These 127 and 117 kDa protein bands shifted back to 120 and 78 kDa products, respectively, after digestion with *N*-glycanase (EC 3.5.1.52) as shown in Fig. 8.

4. Discussion

Our previous reports [11,12] showed that the dialysis method for the liposomal reconstitution of the H^+/peptide transport activity in the renal BBM is more reliable than the freeze/thaw technique. Similarly, in the present study, we found that proteoliposomes derived from the intestinal BBM by the dialysis method are more reliable than the freeze/thaw technique with regard to the H^+-dependency of ceftibuten uptake. It seems that the slow removal of detergent by dialysis permits a safe and proper reconstitution of the transport activity of ceftibuten with characteristics similar to those described in the original brush-border membrane vesicles of rat intestine [24].

In a previous paper [25], we have observed by kinetic analysis that ceftibuten transport is mediated by two transport systems (high- and low-affinity) in the renal brush-border membrane and by one transport system in the intestinal brush-border membrane. However in the present study, SDS/PAGE analysis revealed that two intestinal proteins can interact strongly with ceftibuten as a ligand in affinity chromatography. The apparent molecular masses of these proteins shifted from 117 and 127 kDa to 78 and 120 kDa, respectively, by *N*-glycanase digestion. The rat PepT1 cDNA consists of 2921 bp with an open reading frame encoding a 710 amino acid protein with a calclated molecular mass of 79 kDa, although the molecular size of the PepT1 transport protein core is reported to be ~63 kDa [8]. It was shown that a protein with an apparent molecular mass of 75 kDa is detectable in rat intestinal brush-border membrane by immunoblotting with polyclonal antibodies, raised against the carboxyl termini of rat PepT1 [8]. In contrast, Kramer et al. [26,27] observed by photoaffinity labeling that a 127 kDa polypeptide is a component of the intestinal oligopeptide transport system. Our present results demonstrate that a protein with a molecular size of 127 kDa as well as another protein of 117 kDa (protein core molecular size: 78 kDa) recognize the structure of ceftibuten immobi-

Table 1
Purification levels of the H^+-dependent ceftibuten transport activity from rat intestinal brush-border membrane [a]

Step	Specific activity (pmol/mg protein/s)	Purification (-fold)	Yield (%)
Brush-broader membrane	26.1	1.0	100.0
n-Octylglucoside extract	123.1	4.7	56.3
Affinity chromatography	10651.5	615.0	0.26

[a] 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 in the incubation medium was 1.0 mM. Each data point was expressed by means of three or four measurements.

lized to sepharose beads. It is unlikely that the 3.5 M NaCl-eluted fraction contains any protein related to the intestinal anion transport system because that lactic acid, a typical substrate for the anion transporter, was included in the starting buffer along the affinity chromatography course.

It follows from the foregoing discussion that there are two possibilities for explaining the contradiction of the kinetic observations with those of purification. The first is the supposition that two transport systems are present in the intestine but with very close substrate affinities, which makes the kinetic approach inefficient in distinguishing them. The other possibility is that the intestinal transporter is a heterodimer composed of two subunits, one is the PepT1 (the catalytic subunit), and the other (the regulatory subunit) is the 127 kDa protein isolated by Kramer et al. [26,27]. Finding which of the two possibilities is factual needs further investigation, but we think that our affinity chromatography approach of this study provides a valuable tool for answering the many-sided questions on oligopeptide transport mechanism.

It is noteworthy that the purification protocol presented in this study allowed a 615-fold purification compared with the crude brush-border membrane fraction (Table 1).

Amino acid sequencing and sugar analysis of the isolated protein are in progress.

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