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Isolation and characterization of the outer membrane of *Escherichia coli* with autodisplayed Z-domains

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ABSTRACT

"Autodisplay technology" is an expression technique used to display the various recombinant proteins on the outer membrane (OM) of *Escherichia coli*. The resulting autodisplayed Z-domain has been used to improve the sensitivity of immunoassays. In this work, a facile isolation method of the OM fraction of *E. coli* with autodisplayed Z-domains was presented using (1) an enzyme reaction for the hydrolysis of the peptidoglycan layer and (2) short centrifugation steps. The purity of the isolated OM fraction was analyzed. For the estimation of contamination with bacterial proteins from other parts of *E. coli*, Western blots of marker proteins for the OM (OmpA), periplasm (β-lactamase), inner membrane (SecA), and cytoplasm (β-galactosidase) were performed. Additionally, assays of marker components or enzymes from each part of *E. coli* were carried out including the OM (KDO), inner membrane (NADH oxidase), periplasm (β-lactamase), and cytoplasm (β-galactosidase). The yield of OM isolation using this new method was determined to be 80% of the total OM amount, with less than 1% being contaminants from other parts of *E. coli*.

1. Introduction

The outer surface of gram-negative bacteria, including *Escherichia coli*, is composed of an outer membrane (OM), a peptidoglycan layer, and an inner membrane (IM) [1]. The periplasm (PP) is located between the OM and the IM, which surrounds the cytoplasm (CP) of *E. coli*. The OM of *E. coli* consists of lipopolysaccharides (LPS), lipoproteins, and other outer membrane proteins within a thickness of 7 nm [2]. Recently, we reported on the results of using "autodisplay technology" for the expression of various recombinant proteins on the OM of *E. coli*, such as Z-domain, streptavidin, and Ro/SSA proteins [3–7]. Using this method, the autotransporter domain of AIDA-1, an OM *E. coli* protein for the translocation of a target protein, was expressed using a signal peptide of the cholera toxin subunit (CTB) and an artificial promoter [3,4]. In particular, the autodisplayed Z-domain has also been used in immunoassays for orientation control of antibodies by using its specific binding affinity to the F_c region of IgGs [3–5,8,9]. To apply the autodisplayed Z-domains, two kinds of immunoassay configurations were used: (1) whole *E. coli* cells with autodisplayed Z-domains were directly used for immunoassays after treatment with the detection antibodies [10–13], and (2) the OM with autodisplayed Z-domains was isolated from *E. coli* and then layered on a surface plasmon resonance (SPR) biosensor with fluorescence microarrays and gold electrodes for amperometric analysis [5,11,13–15]. Both immunoassay techniques of the OM layer with autodisplayed Z-domains showed greatly improved sensitivity and limits of detection in comparison with immunoassays using randomly oriented antibodies. Given that these immunoassays based on the OM with autodisplayed Z-domains are usually applied for the medical diagnosis of acute and chronic inflammatory diseases, the contamination of bacterial proteins from other parts of *E. coli* could result in false-positives due to non-specific binding of antibodies in human serum samples [11,16,17].

In this work, we present a facile OM isolation method from *E. coli* with autodisplayed Z-domains using (1) an enzyme reaction for the hydrolysis of the peptidoglycan layer and (2) short centrifugation steps. Then, the purity of the isolated OM fraction was analyzed by (1) Western blot of marker proteins [3,18–20] and (2) an assay of marker enzymes [18,21–23] of each *E. coli* part (OM, PP, IM, and CP).

2. Materials and methods

2.1. Materials

LB broth was bought from Duchefa (Haarlem, Netherlands) and the microplate was purchased from Nunc Co. (Roskilde, Denmark). Mouse monoclonal anti-β-lactamase antibodies, mouse monoclonal anti-histidine tag (His_6) antibodies, mouse monoclonal anti-β-galactosidase antibodies, and rabbit polyclonal anti-mouse IgG antibodies conjugated with HRP were purchased from AbCam Co. (Cambridge, UK). Aprotinin

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was bought from Roche Korea (Seoul, Korea) and luminol substrate was purchased from Thermo Scientific Co. (USA). NADH oxidase and β-lactamase were purchased from Merck Co. (USA). Purpald, 2-keto-3-deoxyoctona (KDO), β-nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), penicillin G, carbenicillin, lysozyme, DNase, and all of the other chemicals (of analytical grade) were purchased from Sigma-Aldrich Korea (Seoul, Korea). A SensoLyte® ONPG β-galactosidase assay kit and a β-galactosidase assay kit were bought from Anaspec Co. (USA).

2.2. OM isolation

The *E. coli* strains of UT5600 (DE3) pT7secA-His and UT5600 (DE3) pET-Z-18-3 were grown overnight in LB medium with carbenicillin (50 μg/mL). After overnight culture, the *E. coli* solution (200 μL) was rinsed with fresh LB media and fresh LB media with antibiotics (carbenicillin, 20 mL) was added. The *E. coli* solution was incubated at 37 °C with vigorous shaking (200 rpm) until the optical density reached $OD_{578\ nm}$ 0.7. Then, Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) was added to cells and the solution was incubated for 1 h at 30 °C with vigorous shaking (200 rpm). The harvested *E. coli* cells were resuspended with 0.2 M Tris–HCl buffer (pH 8.0) and lysed by lysozyme (200 μg/mL in a final concentration with 20 mM sucrose and 0.2 mM EDTA) for 10 min at RT. Then, PMSF (1 mM) and aprotinin (20 μg/mL) were added. The OMs of *E. coli* cells were isolated by the addition of the same volume of extraction buffer (2% Triton X-100, 50 mM Tris–HCl, 10 mM $MgCl_2$) with DNAse (10 μg/mL). After incubation for 30 min on ice, the lysate was centrifuged at 4,000 rpm for 5 min. The OM-containing supernatant was centrifuged at 18,000 rpm for 10 min and washed with PBS 2 times. After washing, the OM was dissolved in the DW for SDS-PAGE analysis and in PBS for the enzymatic assay.

2.3. Analysis of the isolated OM fraction with Western blotting of marker proteins

The isolated OMs were diluted (1:2) with a sample buffer (100 mM Tris–HCl, pH 6.8, containing 4% SDS, 0.2% bromophenol blue, and 20% glycerol). The prepared OM samples were boiled for 20 min and then analyzed using 12.5% SDS-PAGE. After electrophoresis, proteins were visualized by staining with Coomassie brilliant blue. The molecular weight of the OM marker protein, outer membrane protein A (OmpA), was confirmed by comparison with the pre-stained molecular weight markers (Bio-Rad, USA). For Western blotting, proteins on the SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane under an AC voltage of 100 V. The membrane was blocked with TBS containing 0.1% Tween-20 and 5% skim milk at RT for 3 h. Then, the primary antibodies at a concentration of 2 μg/mL were added and incubated at RT for 3 h and the membrane-bound antibodies were stained with 1:10,000 diluted HRP-labeled anti-mouse antibodies. To detect the marker proteins, luminol solution was added. Images of the membranes were taken using a documentation system (Bio-Rad, USA).

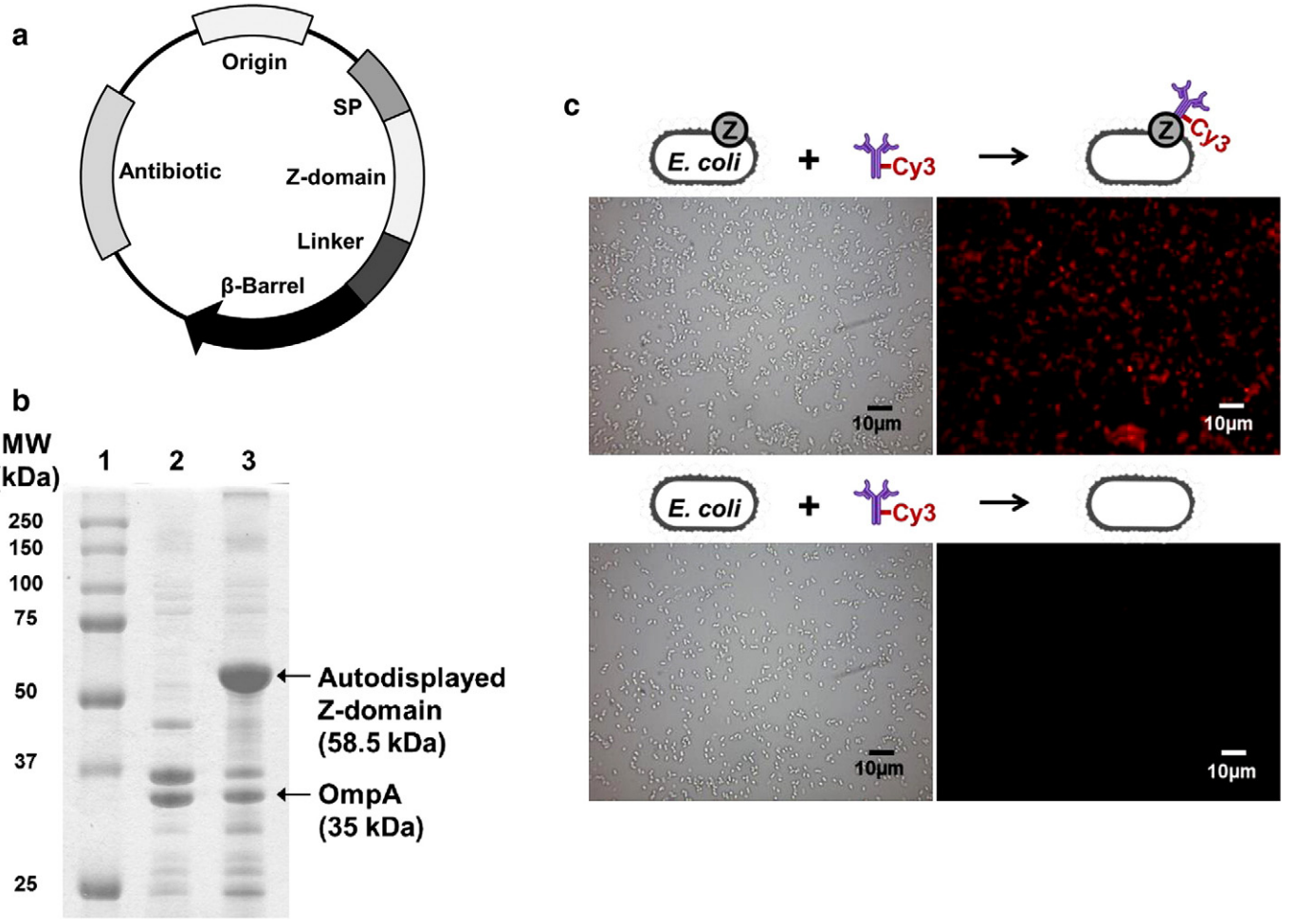

Fig. 1. Autodisplay of Z-domains on the OM of *E. coli*. (a) Structure of the autodisplay vector of Z-domains, (b) SDS-PAGE analysis of OM proteins before and after autodisplay of Z-domains, and (c) fluorescence microscopy image of *E. coli* after treatment with fluorescence (Cy-3)-labeled IgG.

2.4. Analysis of the isolated OM fraction with assays of marker enzymes

The KDO (keto-3-deoxy-D-manno-oct-2-ulosonic acid) assay was carried out for the quantification of LPS [23]. Isolated OM samples were mixed with sodium periodate (32 mM) in a 96-well microplate and incubated at RT for 25 min. After incubation, purpald solution (136 mM) was added and the plate was incubated at RT for 20 min. Then, additional sodium periodate (64 mM) was added and incubated at RT for 20 min. The absorbance was measured at a wavelength of 550 nm with a microplate reader. The activity of β-lactamase was measured according to reference [22]. The penicillin G solution (10 μL) at a concentration of 3 mg/mL was added to the isolated OM solution (190 μL) and the standard β-lactamase samples at RT for 10 min. After incubation, the detection solution which was mixed with the same volume of solution A and solution B (solution A: acetate buffer with pH 4.75 containing 80 mg/mL neocuproine-HCl and 1 g/mL SDS; solution B: acetate buffer with pH 4.75 containing 200 mg/mL copper (II) sulfate and 1 g/mL SDS) was added to the reaction mixture and incubated at RT for 10 min. Then, the absorbance was measured at a wavelength of 550 nm with a microplate reader. The activity of NADH oxidase was measured according to reference [21]. NAD (20 nM) and FAD (10 nM) were mixed with NADH oxidase samples (200 μL) in a 96-well microplate. The plate was incubated at RT for 1 h and the optical density was measured at a wavelength of 340 nm with a microplate reader.

3. Results and discussion

3.1. E. coli OM isolation

The Z-domain was autodisplayed on the OM of *E. coli* by transformation of the Z-domain autodisplay vector (pET-Z-18-3) into UT5600 (DE3). As shown in Fig. 1(a), the autodisplay vector contained the autotransporter domain of the *E. coli* AIDA-1 protein, used for the

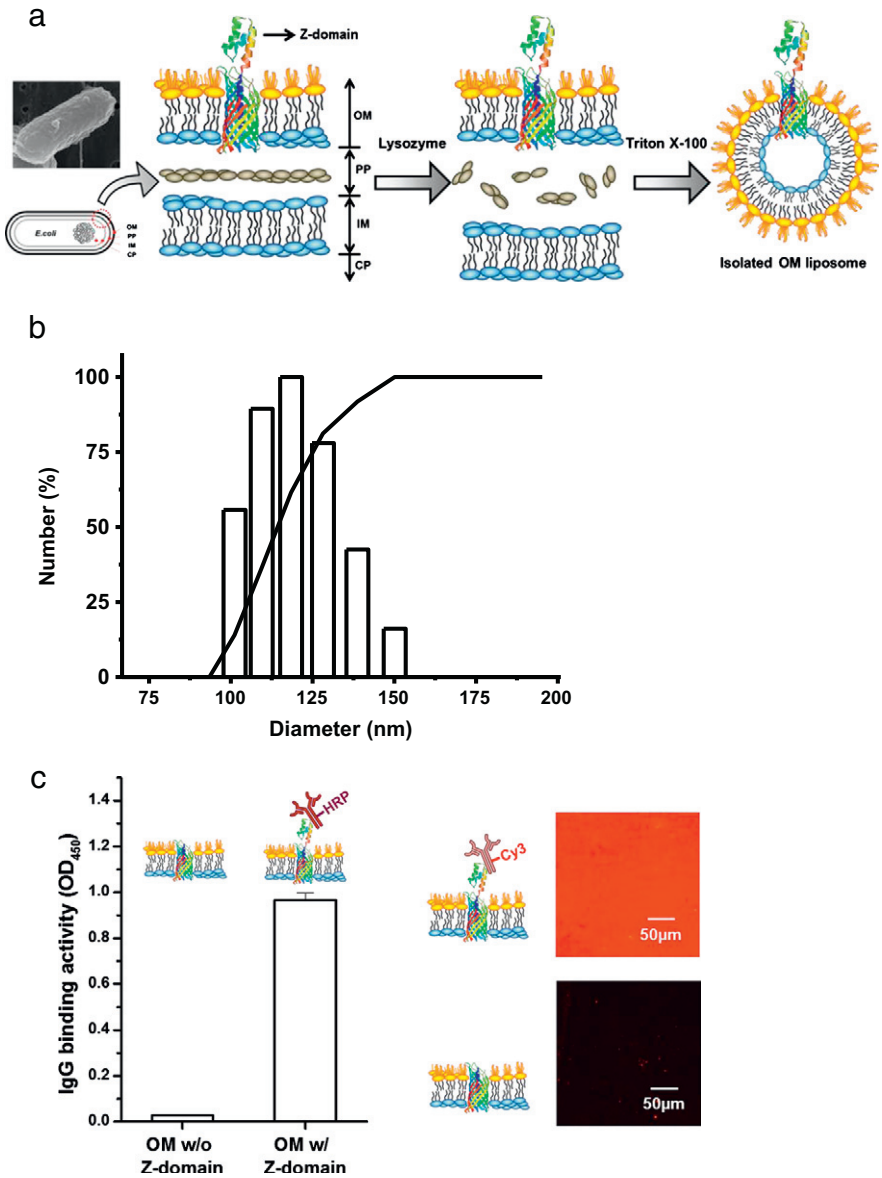

Fig. 2. Isolation of the *E. coli* OM: (a) the OM isolation procedure; (b) the size distribution analysis of the isolated OM liposomes using laser dynamic scattering (LDS), (c) the IgG-binding activity of the OM layers on polystyrene microplates. The OM layer from intact *E. coli* without Z-domain and the OM layer with autodisplayed Z-domains were reacted with HRP-labeled IgG. The amount of bound IgG to the OM layers were quantified using the chromogenic reaction of TMB.

outer membrane translocation of a target protein by using the signal peptide of the cholera toxin subunit (CTB) and an artificial promoter [3,4]. The expression of the Z-domains on the OM of *E. coli* was confirmed by SDS-PAGE of the OM proteins. As shown in Fig. 1(b), Z-domains were observed to be expressed on the outer membrane of *E. coli* with a molecular weight of 58.5 KDa. In the next step, the autodisplayed Z-domains were tested to determine IgG-binding activity by treatment with fluorescence (Cy-3)-labeled IgG. As shown in Fig. 1(c), the *E. coli* with autodisplayed Z-domains presented strong fluorescence because the autodisplayed Z-domains could bind the fluorescence (Cy-3)-labeled IgG. However, the intact *E. coli* without the autodisplayed Z-domains were observed to have no significant fluorescence. These results suggested that the Z-domains were indeed autodisplayed on the *E. coli* OM and had IgG-binding activity.

In this work, the isolation of the OM fraction of *E. coli* was carried out using a modified method from Hantke et al. [24]. The OM fraction was isolated by (1) enzyme treatment for the hydrolysis of the peptidoglycan layer with lysozymes and Triton X-100 and (2) short centrifugation steps, as shown in Fig. 2(a). After the enzyme treatment, the OM was isolated as liposomes using Triton X-100 as a detergent [25]. The OM liposomes were isolated from the cell lysate of *E. coli* by a centrifugation at 18,000 rpm for 10 min. As shown in Fig. 2(b), the liposome of OM was observed to have a homogeneous size in a diameter range of 100–150 nm using a dynamic laser scattering method (DLS) [26]. The recovery of the OM isolation was calculated from the KDO amount of *E. coli* and the isolated OM. As each *E. coli* cell was reported to have 2×10^6 LPS and each LPS had two KDO, the total number of KDO in each *E. coli* cell was estimated to be 4×10^6 [27]. In this work, the number of KDO in the isolated OM per each *E. coli* was estimated to be 1.5×10^6 and the recovery rate of the isolated OM was estimated to be approximately 37.5 %. The OM layer was transferred onto a polystyrene microplate through the hydrophobic interaction between the hydrophobic core and the surface of the microplate [5]. The IgG-binding activity of the OM layer with autodisplayed Z-domains was estimated by the treatment of IgG with horseradish peroxidase (HRP) and the chromogenic reaction with 2,2′,5,5′-tetramethylbenzidin (TMB). As shown in Fig. 2(c), the OM layer with autodisplayed Z-domains showed a far higher IgG-binding activity in comparison with the OM layer from the intact *E. coli* without autodisplayed Z-domains. These results indicated that the OM layer with autodisplayed Z-domains could be formed on the microplate through hydrophobic interactions and the IgG-binding activity could be maintained.

3.2. Analysis of isolated E. coli OM with marker proteins

As previously mentioned, the contamination of the isolated OM fraction with bacterial proteins from other parts of *E. coli* should be minimized for its application in immunoassays in order to avoid false-positive results from non-specific binding of antibodies in human serum samples. In this work, the purity of the isolated OM fraction was analyzed through Western blotting of marker proteins for the OM, PP, IM, and CP. OmpA and SecA were selected as major proteins of the OM and IM of *E. coli*, respectively [3,19]. β-Lactamase and β-galactosidase were selected as marker proteins of the PP and CP of *E. coli*, respectively [18,20]. For the effective analysis of SecA, β-lactamase, and β-galactosidase, a plasmid (pT7secA-His) with a β-lactam-resistant gene was transformed into UT5600(DE3) *E. coli* for the expression of (1) SecA with a 6× his tag, (2) β-lactamase, and (3) β-galactosidase [28].

As shown in Fig. 3(a), OmpA was directly analyzed in the SDS-PAGE with Coomassie brilliant blue staining [3,29,30]. Although the protein band of OmpA (35 kDa) was observed for isolated OM fractions (lane 3 and 4) as well as at the whole cell lysate (lanes 1 and 2), the relative amount of OmpA was estimated to be far higher in the isolated OM fractions than in the whole cell lysate samples. These results indicated that the OM proteins were well-isolated in the OM fractions from the whole cell lysate.

For the detection of other marker proteins of β-lactamase (PP), SecA with a 6× his tag (IM) and β-galactosidase (CP), Western blotting was carried out by treatment with anti-β-lactamase antibodies (monoclonal), anti-6× his tag antibodies (monoclonal), and anti-β-galactosidase antibodies (monoclonal), respectively. Then, the chemiluminescence images of marker protein bands were taken after the addition of luminol solution.

As shown in Fig. 3(b), the β-lactamase (a marker protein of PP, 31.5 kDa) was detected in the whole cell lysate of *E. coli*, which was transformed with the pT7-SecA plasmid (lane 2), and the cell whole cell lysate of the intact *E. coli* showed no β-lactamase protein band (lane 1). The isolated OM fractions from both *E. coli* strains also showed no β-lactamase protein band (lanes 3 and 4). These results indicated that the isolated OM fraction was not contaminated with the bacterial proteins from the PP.

In the case of SecA (a marker protein of the IM, 102 kDa) and β-galactosidase (a marker protein of the CP, 116 kDa), both proteins were only observed in the whole cell lysate of *E. coli* which was transformed with a pT7secA-His plasmid (lane 2). However, the cell whole cell lysate of intact *E. coli* as well as the isolated OM fractions showed no β-lactamase protein band (lane 1). These results indicated that the isolated OM fraction was not contaminated with the bacterial proteins from the IM and CP. Based on these Western blot results, the OM fraction in this work was determined to be isolated without the contamination of proteins from the PP, IM, and CP.

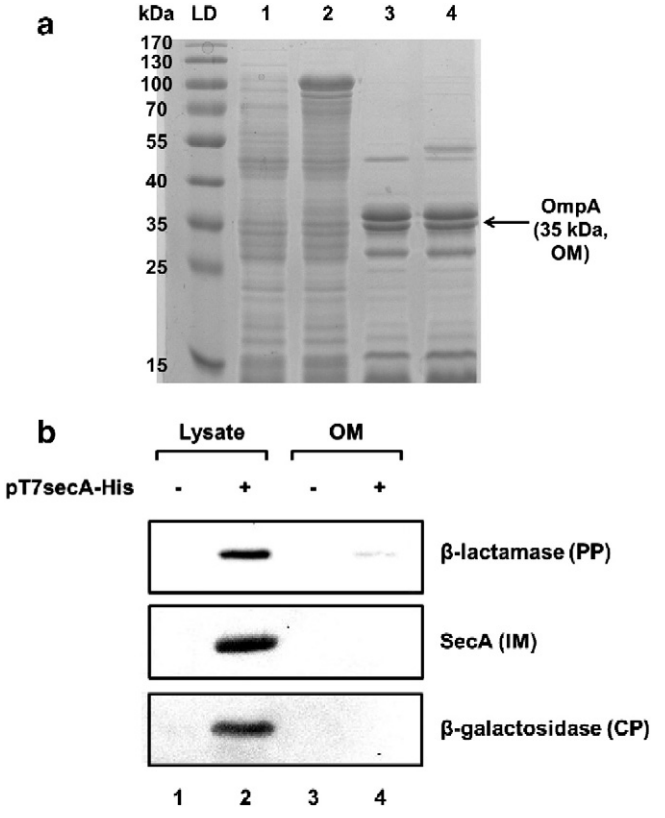

Fig. 3. Analysis of the isolated *E. coli* OM with SDS-PAGE and Western blotting. (a) SDS-PAGE of the isolated *E. coli* OM (lane 1: whole cell lysate of intact *E. coli*; lane 2: whole cell lysate of transformed *E. coli* with pT7secA-His; lane 3: OM fraction from intact *E. coli*; lane 4: OM fraction from transformed *E. coli*). OmpA (a marker protein of the OM) was observed in the OM fractions (lanes 3 and 4) after Coomassie staining. (b) Western blotting of β-lactamase (a marker protein of the PP), SecA (a marker protein of the IM), and β-galactosidase (a marker protein of the CP).

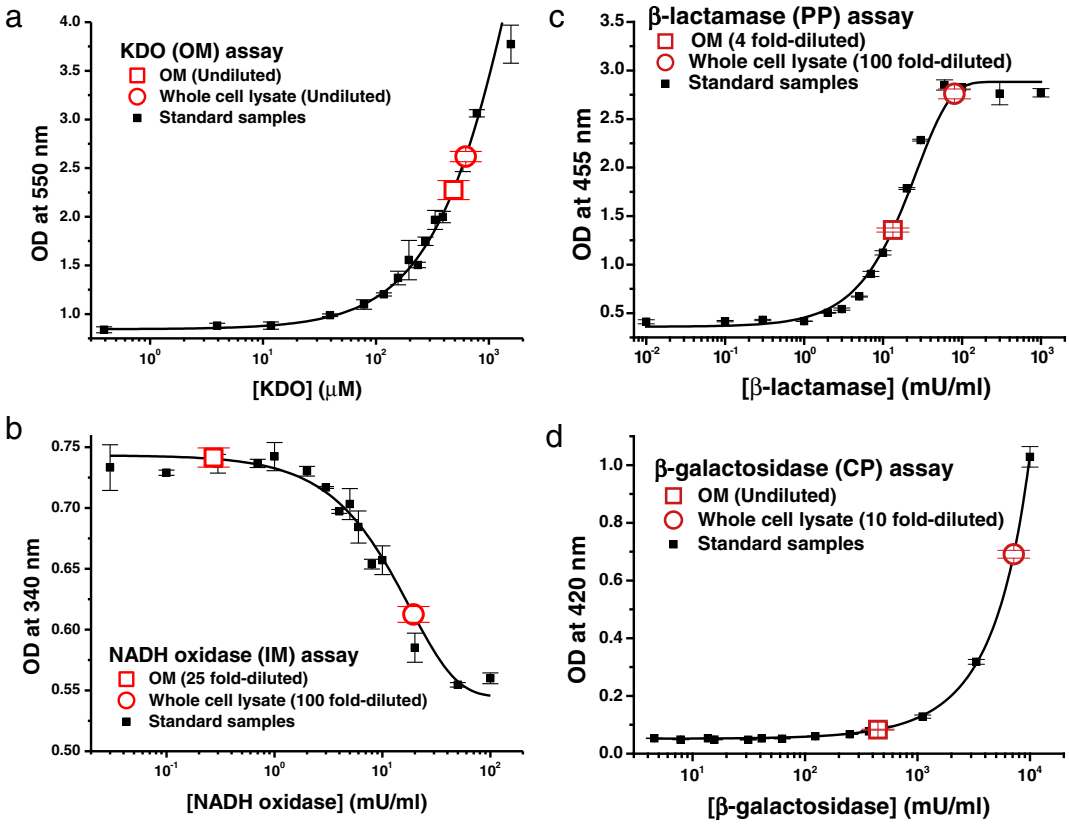

Fig. 4. Characterization of the isolated OM with marker enzymes from each *E. coli* part (OM, PP, IM, and CP). (a) KDO, (b) NADH oxidase, (c) β-lactamase, (d) β-galactosidase assays as marker enzymes for the OM, IM, PP and CP, respectively.

3.3. Analysis of isolated E. coli OM with enzyme assays

For the quantitative determination of the purity of the isolated OM fraction of *E. coli* with autodisplayed Z-domains, the marker enzymes for each part of *E. coli* (OM, PP, IM, and CP) were assayed. KDO, NADH oxidase, β-lactamase, and β-galactosidase were selected for the quantitative analysis of the OM, IM, PP, and CP, respectively [18,21–23].

Lipopolysaccharide (LPS) was selected as a marker component of the OM fraction of *E. coli*, which is one of the major components of the OM [31]. For the quantitative analysis of the OM isolation yield of this method, the amount of LPS was determined using a KDO assay at a concentration range of 0.4–1600 μM [23]. As shown in Fig. 4(a), the LPS concentration of the isolated OM fraction and the whole cell lysate (including the OM) was determined to be 485 and 624 μM, respectively. These results showed that this isolation method could isolate approximately 80% of the OM.

In the case of β-lactamase (a marker enzyme of the PP), the assay was carried out at the concentration range of 0.01–1000 mU/mL. As shown in Fig. 4(b), the β-lactamase activity of the isolated OM and the cell lysate (including OM) was determined to be 0.05 and 7.99 mU/mL, respectively. These results showed that the isolated OM fraction contains less than 1% (0.6%) PP contamination. The NADH oxidase was selected as a marker enzyme of the IM [21]. As shown in Fig. 4(c), the NADH oxidase activity of the isolated OM and the whole cell lysate (including OM) was determined to be 0.01 and 1.94 mU/mL, respectively. These results showed that the isolated OM fraction contains less than 1% (0.5%) IM contamination. The β-galactosidase assay (a marker enzyme of the CP) was carried out by using a commercial assay kit from Anaspec Co. (USA) at a concentration range of 5–10,000 mU/mL. As shown in Fig. 4(d), the of β-galactosidase activity of the isolated OM and the cell lysate (including OM) was determined to be 0.44 and 71.76 mU/mL, respectively. These results showed that the isolated OM fraction contains less than 1% (0.6%) CP contamination. These results indicate that the yield of the OM isolation with this new method was 80% of the total OM amount with less than 1% contamination from the other parts of *E. coli* (the PP, IM, and CP) as summarized in Table 1.

Table 1
The activity of marker enzymes of *E. coli* compartments in the isolated OM.

Assay (*E. coli* compartment)	Activity value		Relative activity in isolated OM
	Whole cell lysate	Isolated OM	
KDO (OM)	624 μM	485 μM	80%
NADH oxidase (IM)	7.99 mU/mL	0.05 mU/mL	0.6%
β-Lactamase (PP)	1.94 mU/mL	0.01 mU/mL	0.5%
β-galactosidase (CP)	71.76 mU/mL	0.44 mU/mL	0.6%

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References

[1] H. Tokuda, S. Matsuyama, Sorting of lipoproteins to the outer membrane in *E. coli*, Biochim. Biophys. Acta, Mol. Cell Res. 1693 (2004) 5–13.
[2] M. Inouye, 3-Dimensional molecular assembly model of a lipoprotein from *Escherichia coli* outer membrane, Proc. Natl. Acad. Sci. U. S. A. 71 (1974) 2396–2400.
[3] J. Jose, J.W. Chung, B.J. Jeon, R.M. Maas, C.H. Nam, J.C. Pyun, *Escherichia coli* with autodisplayed Z-domain of protein A for signal amplification of SPR biosensor, Biosens. Bioelectron. 24 (2009) 1324–1329.

[4] J. Jose, M. Park, J.C. Pyun, E-coli outer membrane with autodisplayed Z-domain as a molecular recognition layer of SPR biosensor, Biosens. Bioelectron. 25 (2010) 1225–1228.
[5] M. Park, J. Jose, J.C. Pyun, Hypersensitive immunoassay by using *Escherichia coli* outer membrane with autodisplayed Z-domains, Enzym. Microb. Technol. 46 (2010) 309–314.
[6] K. Petermann, S. Vordenbaumen, J.C. Pyun, A. Braukmann, E. Bleck, M. Schneider, J. Jose, Autodisplay of 60-kDa Ro/SS-A antigen and development of a surface display enzyme-linked immunosorbent assay for systemic lupus erythematosus patient sera screening, Anal. Biochem. 407 (2010) 72–78.
[7] G. Yoo, J.H. Bong, S. Kim, J. Jose, J.C. Pyun, Microarray based on autodisplayed Ro proteins for medical diagnosis of systemic lupus erythematosus (SLE), Biosens. Bioelectron. 57 (2014) 213–218.
[8] J.C. Pyun, S.D. Kim, J.W. Chung, New immobilization method for immunoaffinity biosensors by using thiolated proteins, Anal. Biochem. 347 (2005) 227–233.
[9] J.W. Chung, J.M. Park, R. Bernhardt, J.C. Pyun, Immunosensor with a controlled orientation of antibodies by using NeutrAvidin-protein A complex at immunoaffinity layer, J. Biotechnol. 126 (2006) 325–333.
[10] J. Jose, M. Park, J.C. Pyun, Highly sensitive immunoassay based on *E. coli* with autodisplayed Z-domain, Anal. Chim. Acta. 667 (2010) 113–118.
[11] M. Park, J. Jose, J.C. Pyun, SPR biosensor by using *E. coli* outer membrane layer with autodisplayed Z-domains, Sensors Actuators B Chem. 154 (2011) 82–88.
[12] M. Park, J.H. Bong, G. Yoo, J. Jose, M.J. Kang, J.C. Pyun, Flow cytometric immunoassay using *E. coli* with autodisplayed Z-domains, Enzym. Microb. Technol. 53 (2013) 181–188.
[13] G. Yoo, J.H. Bong, M. Park, M.J. Kang, J. Jose, J.C. Pyun, Magnetic-bead-based immunoassay using *E. coli* cells with autodisplayed Z-domains, Enzym. Microb. Technol. 53 (2013) 118–122.
[14] J.-H. Bong, G. Yoo, M. Park, M.-J. Kang, J. Jose, J.-C. Pyun, Ultrasonic isolation of the outer membrane of *Escherichia coli* with autodisplayed Z-domains, Enzym. Microb. Technol. 66 (2014) 42–47.
[15] J. Oh, G. Yoo, Y.W. Chang, H.J. Kim, J. Jose, E. Kim, J.C. Pyun, K.H. Yoo, A carbon nanotube metal semiconductor field effect transistor-based biosensor for detection of amyloid-beta in human serum, Biosens. Bioelectron. 50 (2013) 345–350.
[16] J.K. Lee, G. Yoo, M. Park, J. Jose, M.J. Kang, J.C. Pyun, Electrochemical ELISA based on *Escherichia coli* with autodisplayed Z-domains, Sensors Actuators B Chem. 175 (2012) 46–52.
[17] M. Park, J.H. Bong, Y.W. Chang, G. Yoo, J. Jose, M.J. Kang, J.C. Pyun, FACS-based immunoassay of troponin-I using *E. coli* cells with autodisplayed Z-domains, Anal. Methods 6 (2014) 1700–1708.
[18] G.R. Jacobson, B.J. Takacs, J.P. Rosenbusch, Properties of a major protein released from *Escherichia coli* by osmotic shock, Biochemistry 15 (1976) 2297–2303.
[19] Y.J. Kim, T. Rajapandi, D. Oliver, SecA protein is exposed to the periplasmic surface of the *E. coli* inner membrane in its active state, Cell 78 (1994) 845–853.
[20] D.M. Long, Enzymatic assay of beta-lactamase using circular dichroism spectropolarimetry, Anal. Biochem. 247 (1997) 389–393.
[21] V.M. Reusch, M.M. Burger, Distribution of marker enzymes between mesosomal and protoplast membranes, J. Biol. Chem. 249 (1974) 5337–5345.
[22] M.A. Cohenford, J. Abraham, A.A. Medeiros, A colorimetric procedure for measuring beta-lactamase activity, Anal. Biochem. 168 (1988) 252–258.
[23] C.H. Lee, C.M. Tsai, Quantification of bacterial lipopolysaccharides by the purpald assay: measuring formaldehyde generated from 2-keto-3-deoxyoctonate and heptose at the inner core by periodate oxidation, Anal. Biochem. 267 (1999) 161–168.
[24] K. Hantke, Regulation of ferric iron transport in Escherichia-Coli-K12—isolation of a constitutive mutant, Mol. Gen. Genet. 182 (1981) 288–292.
[25] C.A. Schnaitman, Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100, J. Bacteriol. 108 (1971) 545–552.
[26] H. Harashima, Y. Midori, S. Ohshima, K. Yachi, H. Kikuchi, H. Kiwada, Kinetic analysis of tissue distribution of doxorubicin incorporated in liposomes in rats (II), Biopharm. Drug Dispos. 14 (1993) 595–608.
[27] P.O. Magalhaes, A.M. Lopes, P.G. Mazzola, C. Rangel-Yagui, T.C.V. Penna, A. Pessoa, Methods of endotoxin removal from biological preparations: a review, J. Pharm. Pharm. Sci. 10 (2007) 388–404.
[28] M.O. Schmidt, R.M. Brosh, D.B. Oliver, *Escherichia coli* SecA helicase activity is not required in vivo for efficient protein translocation or autogenous regulation, J. Biol. Chem. 276 (2001) 37076–37085.
[29] E. Schultheiss, S. Weiss, E. Winterer, R. Maas, E. Heinzle, J. Jose, Esterase autodisplay: enzyme engineering and whole-cell activity determination in microplates with pH sensors, Appl. Environ. Microbiol. 74 (2008) 4782–4791.
[30] M. Park, J. Jose, S. Thommes, J.I. Kim, M.J. Kang, J.C. Pyun, Autodisplay of streptavidin, Enzym. Microb. Technol. 48 (2011) 307–311.
[31] C.F. Earhart, M. Lundrigan, C.L. Pickett, J.R. Pierce, *Escherichia coli* K-12-mutants that lack major outer-membrane protein-A, FEMS Microbiol. Lett. 6 (1979) 277–280.