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Abstract

To explore virus-like particles (VLPs) formation of dengue virus serotype type 2 (DENV-2) structural proteins of, C, prM, E were expressed in silkworm larvae using recombinant *Bombyx mori* nucleopolyhedroviruses (BmNPV). Each recombinant BmNPV bacmid coding the 2C-prM-E polypeptide and E protein fused with the signal peptide of bombyxin from *B. mori* was injected into silkworm larvae. The expressed proteins were collected from hemolymph and fat body, and purified using affinity chromatography. E protein was observed at 55 kDa. The DENV virus-like particles (DENV-LPs) with a diameter approximately 35 nm was observed using transmission electron microscopy (TEM) and immunogold-labelling TEM analysis. The binding of each partially purified proteins to heparin, one of receptors for DENV was confirmed. DENV-LPs were secreted in silkworm larval hemolymph even still low amount, but the E protein and heparin binding function were confirmed.

Keywords Bacmid · Capsid · Dengue virus · Premembrane · Envelope · Silkworm.

Introduction

Dengue virus (DENV) is a positive-stranded RNA virus belongs to Flaviviruses and has four serotypes (DENV 1–4) [1] and infection to human is mediated by *Aedes* mosquitoes. In preventing of DENVs infection, many efforts are devoted on developing the anti-viral substances or vaccines. No anti-viral are available except Dengvaxia by Sanofi Pasteur [2]. This live-attenuated tetravalent vaccine does not show an immune response equally against four DENV serotypes. Since the risk of antibody-dependent enhancement also found in sero-negatives [1], tetravalent vaccines development is needed.

Instead of live-attenuated and inactivated vaccines, recombinant subunit proteins and virus-like particle (VLP)-based vaccines have been developed. Capsid (C), membrane precursor (prM) and envelope (E) of structural proteins of DENV were expressed as a polypeptide before its processing [3]. Especially, E protein is regarded as a target for vaccine development because it is exposed on the viral surface [4]. N-terminal domain of E protein (80E) that lacks of two transmembrane domains was expressed in *Drosophila* S2 cells and secreted into the culture supernatant [5]. DENV-like particles (DENV-LPs) were produced in insect cells and mammalian cells when prM and E proteins were coexpressed as a single polypeptide [6]. In the case of arboviruses, coexpression of prM and E protein led the production of its VLPs in yeasts, insect cells and mammalian cells [7]. In yeasts, some DENV-LPs composed of E proteins were prepared, but these VLPs formed in vitro after its purification from the lysate of *Pichia pastoris* under the denature condition even though the transmembrane domains remain in its C-terminus [8], but was secreted into the culture supernatant in insect cells [5]. It indicates that these VLPs only contain E protein and their morphology may be different with the virions. These VLPs often circumvent the use of an adjuvant in its immunization because it can induces the

host's immunity as well as an adjuvant [9].

In this study, we tried to express DENV type 2 structural proteins in silkworm larvae, since the post-translational modifications are needed to make a proper folding of capsid protein for VLP forming, using recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid.

Materials and methods

Construction of Recombinant BmNPVs

The gene coding DENV serotype 2 (DENV-2) C-prM-E polypeptide (GenBank: M29095.1) was synthesized by Genewiz (New Jersey, USA). The gene coding the C-prM-E polypeptide fused with or without a linker sequence (GGGGSGGGGS) and FLAG tag sequence (DYKDDDDK) at its C-terminus was amplified by PCR using primers set (C-prM-E-F and E-R, Table 1) and a template as the gene coding DENV-2 C-prM-E polypeptide (Fig. 1A). The gene coding E protein fused with the signal peptide of silkworm bombyxin and the linker-FLAG tag sequence at its N- and C-terminus, respectively (Fig. 1B), was amplified by PCR using primers set (E-F and E-R, Table 1) and a template as the gene coding DENV-2 C-prM-E polypeptide. Both constructs have linker and FLAG tag sequences at its C-terminus. Each gene was inserted into pFastbac1 (Thermo Fisher Scientific K. K., Tokyo, Japan) and pFastbac 1/2C-prM-E and /2E were obtained, following by transformed into *Escherichia coli* BmDH10bac CP^- Chi^- [10]. These recombinant BmNPV bacmids were extracted from white colonies after its transformation and designated as BmNPV/2C-prM-E and BmNPV/2E bacmids,

respectively. Each recombinant BmNPV bacmid was injected into silkworm larvae with DMRIE-C (Thermo Fisher Scientific K. K.) and its hemolymph was collected from the larvae after 6–7 d post-injection. A 1-phenyl-2-thiourea was added into the hemolymph at 200 mM for 50 µl per silkworm and the hemolymph was used as a recombinant BmNPV solution. Aliquots of the hemolymph was kept at –80°C before use.

Expression and Purification of 2C-prM-E Polypeptide and 2E Protein in Silkworm Larvae

The hemolymph was diluted with phosphate-buffered saline (PBS, pH 7.4) by 100-fold and injected into fifth instar silkworm larvae (Ehimesansyu, Ehime, Japan) and these larvae were reared for 4 d at 26°C and relative humidity (70–85%) with an artificial diet (Silkmate S2, Nosan Co., Yokohama, Japan). Hemolymph and fat body were collected. One hundred mg of fat body was suspended with 1 ml Tris-buffered saline (TBS, pH 7.6) containing 0.1% TritonX-100 (TBST). For the purification of both proteins, the hemolymph was diluted with 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl by 10-fold and applied into DDDDK-tagged Protein PURIFICATION GEL (MEDICAL & BIOLOGICAL LABORATORIES, Nagoya, Japan) column chromatography. The column was washed by 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl and proteins were eluted with 0.17 M Glycine-HCl buffer (pH 2.3). The eluents were immediately neutralized with 1 M Tris-HCl (pH 8.0). The eluents were concentrated by ultrafiltration using Amicon Ultra-0.5 mL Centrifugal Filter Unit with Ultracel-30K membrane (Merck Japan, Tokyo, Japan). Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific K. K.).

SDS-PAGE and Western Blot

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% or 12% acrylamide for subjecting western blotting. After SDS-PAGE, the proteins were blotted onto a polyvinylidene fluoride membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Five percent skim milk in TBST (pH 7.6) was used for blocking agent. After the blocking the membrane was incubated in 3000-fold diluted rabbit anti-DENV E protein antibody (GeneTex, Irvine, CA, USA) for 1 h. Alternatively, 10000-fold diluted rabbit anti-DENV prM antibody (GeneTex) was used as a primary antibody. After the membrane was washed trice with TBST, it was incubated in 10000-fold diluted horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (MEDICAL & BIOLOGICAL LABORATORIES) for 1 h. Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Merck Japan). Specific bands were detected on a Fluor-S MAX Multi-Imager (Bio-Rad).

Transmission Electron Microscopy (TEM) and Immunoelectron Microscopy (IEM)

To investigate the size and morphology of the expressed 2C-prM-E and 2E proteins using TEM, 20–30 µl of purified proteins was applied on the Cu-grid TEM (Nisshin EM Co., Ltd., Tokyo) and incubated in room temperature for 30 sec. The Cu-grid was washed by 30 µl PBS and incubated in room temperature for 30 sec, repeated 3 times. For negative staining, 2% of phosphotungstic acid (PTA) 30 µl was used on the Cu-grid, incubated for

30 sec in room temperature, and dried in room temperature for overnight. These Cu-grids were analyzed.

For IEM analysis, 20–30 µl of purified proteins was applied on the Cu-grid TEM and incubated in room temperature for 30 sec. The Cu-grid was washed by 30 µl PBS and incubated in room temperature for 30 sec, repeated 3 times. The Cu-grid was blocked with 30 µl of 2% (v/v) BSA in room temperature for 5 min. 30 µl PBS was used to wash the Cu-grid and incubated in room temperature for 30 sec, repeated 3 times. Thirty µl of anti-E rabbit polyclonal antibody (Wako Pure Chemicals) as primary antibody, diluted 100 times with PBS was applied on the Cu-grid and incubated for 2 h in room temperature, followed by washing with 30 µl PBS and incubated in room temperature for 30 sec for 3 times. Thirty µl of goat anti-rabbit IgG-conjugated gold nanoparticles (Wako Pure Chemicals), diluted 50 times with PBS was applied on the Cu-grid and incubated for 2 h in room temperature. Thirty µl PBS was used to wash the Cu-grid and incubated in room temperature for 30 sec, repeated 3 times. Thirty µl of 2% PTA was applied on the Cu-grid, incubated for 30 sec in room temperature, and dried in room temperature for overnight. The Cu-grid was analyzed using TEM.

Heparin Binding Assay

Avidin Plate Blocking-less type (Sumitomo Bakelite, Tokyo, Japan) were used as an ELISA plate. Biotin-labeled heparin (Sigma-Aldrich Japan, Tokyo, Japan) was diluted with PBS containing 0.05% Tween 80 to 6 ng/ml and the 1.8 ng of heparin was immobilized onto the plates by the incubation at room temperature for 1 h. After the immobilization, wells were washed with PBS containing 0.05% Tween 80 three times.

Purified proteins were added into wells at each amount and the plates were incubated at room temperature for 1 h. After the wash of each wells with PBS containing 0.05% Tween 80, mouse anti-DDDDK tag antibody (MEDICAL & BIOLOGICAL LABORATORIES) diluted by 1000-fold was added into each well and the plates were incubated at room temperature for 1 h, followed by washing the well with PBS supplemented with Tween 20 (PBST). Next, HRP-conjugated anti-mouse IgG antibody (MEDICAL & BIOLOGICAL LABORATORIES) diluted with by 1000-fold was added into each well and the plates were incubated at room temperature for 1 h, followed by washing the well with PBST. One hundred microliters of 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine in 100 mM sodium acetate (pH 6.0) with 0.2% (v/v) of 30% H_2O_2 was added to each well and incubated at room temperature. When a blue color developed, the HRP reaction was stopped by the addition of 50 µl of 1 N H_2SO_4 solution, and the absorbance of each well was measured at 450 nm.

In other ELISA, 2 mg of partially purified proteins was mixed with each amount of free heparin and added into the well immobilized with biotin-labeled heparin. The binding was detected in the same protocol described above.

Results

Expression of C-prM-E Polypeptide and E Protein in Silkworm Larvae

Structural proteins and non-structural proteins of DENV2 were expressed as a single polypeptide and processed by host's proteases and its own protease. Therefore, we tried to express the structural proteins, C, prM and E as a single polypeptide in silkworm larvae

and pupae using recombinant BmNPV bacmid under the control of polyhedrin promoter.

The E protein was detected by anti-DDDDK at its estimated molecular weight 55 kDa in both hemolymph (Fig. 2A) and pupae (Fig. 2B) of silkworm. When C-prM-E was expressed in silkworm larvae the E protein was also detected by anti-E antibody in the hemolymph (Fig. 2C) and in silkworm pupae (Fig. 2D). These results indicate that C-prM-E polypeptide was processed to E protein by intrinsic proteases in silkworm larvae and secreted into the hemolymph. In Bm5 cells, the same results were obtained when E protein and C-prM-E polypeptide were expressed (Fig. 2E).

Purification and Characterization of E Protein and C-prM-E Polypeptide from the Hemolymph

The expressed E protein from the hemolymph was purified using the affinity chromatography. The eluent of each construct from DDDDK-tagged protein purification gel had the clear band observed at the molecular weight of E protein (Fig. 3A, B). Fig. 3C and D showed the concentrated eluent from each construct around 55 kDa. Partially purified amount of E protein and C-prM-E polypeptide from 5 ml of hemolymph were 278 and 397 μg, respectively (Fig. 3E).

Morphology of E Protein and C-prM-E Polypeptide

TEM and IEM were performed to confirm its shape and size of the expressed proteins. Spherical particles with a diameter about 35 nm was observed (Fig. 4A, C). The spherical structures shown in TEM analysis bound to gold nanoparticles (Fig. 4B, D).

Accumulation of gold nanoparticles on the spherical structures suggests that these particles are DENV-LPs.

Binding Assay to Heparin

We investigated the binding capacity of each partially purified proteins to heparin by ELISA. In this ELISA, biotin-labeled heparin was used as a ligand immobilized onto an avidin-coated plate. As the concentration of partially purified E (Fig. 5A) and C-prM-E (Fig. 5B) increased, higher absorbance was observed. BSA as a negative control was not affected on the binding assay. When heparin was added in the binding assay the absorbance was decreased for E (Fig. 5C) and C-prM-E (Fig. 5D). These results indicate that the E protein and C-prM-E polypeptide from hemolymph were functional.

Discussion

In the case of arbovirus VLP expression, these VLPs were secreted extracellularly as a particle when only E protein or prM-E polypeptide is expressed in mammalian and insect cells [11]. When C-prM-E polypeptide was expressed in silkworm larvae, the E protein was detected in the hemolymph. After its purification, E protein also detected in the eluents with a band at around 55 kDa. This result suggests that C-prM-E polypeptide may form VLPs because these structural proteins were secreted into hemolymph. It was previously reported that replacement of C-terminus of E protein with that of other arbovirus, Japanese encephalitis virus, enhanced the secretion of its E protein in mammalian cells [12] because the C-terminus of DENV E protein more strongly forces

the retention in endoplasmic reticulum (ER) by its ER retention signal at its C-terminus than that of original E protein [13]. It might possible to improve the generation of DENV-LP in silkworm larvae by replacing this DENV-2 C-terminal domain with Japanese encephalitis C-terminus of E protein.

In mammalian cells, the C-prM-E polypeptide of the DENV was clearly processed into C, prM and E proteins [13]. In insect cells and yeasts, prM-E polypeptide was also cleaved into prM and E protein, but the processing of C-prM-E polypeptide was unknown [12]. In yeasts, processed E protein was detected when C-prM-E protein was expressed [14]. Processing of C-prM-E polypeptide to prM and E protein was carried out by host's signal peptidases and C is released by a viral serine protease (NS3) [4]. In this study, E protein was detected at its estimated molecular weight in silkworms, but prM was detected at its larger molecular weight than estimated. These results indicate that C-prM-E polypeptide was processed into C-prM polypeptide and E protein in silkworms. C-prM and prM-E polypeptides are cleaved by host's signal peptidases, but, in silkworms, C-prM polypeptide was not cleaved. It is possible that different signal peptidases work to C-prM and prM-E proteins, respectively, or conformational restriction causes the difference of its processing. Regarding the latter, prM protein of Murray Valley encephalitis virus was processed by a signal peptidase after the cleavage of C protein by viral NS3 protease [15].

In DENV, the C protein which forms nucleocapsid-like particles, induced the immune response in mice by its immunization [16]. In order to form VLPs, however, prM/M complexes are needed which stabilize E epitope in the acidic environment, even though E protein dengue soluble alone is enough to bind the cells and cause neutralization antibodies [17]. For E protein to achieve its final conformation, an interaction with prM

is necessary, followed by creation of a hetero-oligomeric structure. On the opposite, even with lack of E protein, the final confirmation of prM seems to be possible [18].

The formation of VLP was found in both constructs which have a smaller size, approximately 35 nm, compare to natural virus particles (40–60 nm). The maturation of VLPs may affects epitope display of envelope on the surface of VLPs which may lead to less recognition by anti-E polyclonal (Fig. 3B, D) [19]. Further studies need to be done to analyze the maturity and functionality of the VLPs using animal experiments and against DENV-2.

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Conflict of interests All authors declare that they have no competing interests.

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Table 1. Used primers

Name	5’–3’
C-prM-E-F	TAATCTCGAGATGAATAACCAACGAAAAAAGGCGAGAA ATACG
E-R	TAATGGTACCTTACTTGTCGTCATCGTCTTTGTAGTCGCTTCC ACCTCCACCGCTTCCACCTCCACCGCTTCCACCTCCACCGG CCTGCACCATAACTCCCAAATACAGCG
E-F	GCCTCTAGAATGAAGATACTCCTTGCTATTGCATTAATGTTGT CAACAGTAATGTGGGTGTCAACACAAATGCGTTGCATAGGA

Figure legends

Fig. 1 Construction of recombinant DENV structural proteins expressed in this study. C-prM-E polypeptide (A) and E protein (B) of DENV2 were expressed in silkworms and Bm5 cells as a fusion protein with FLAG tag.

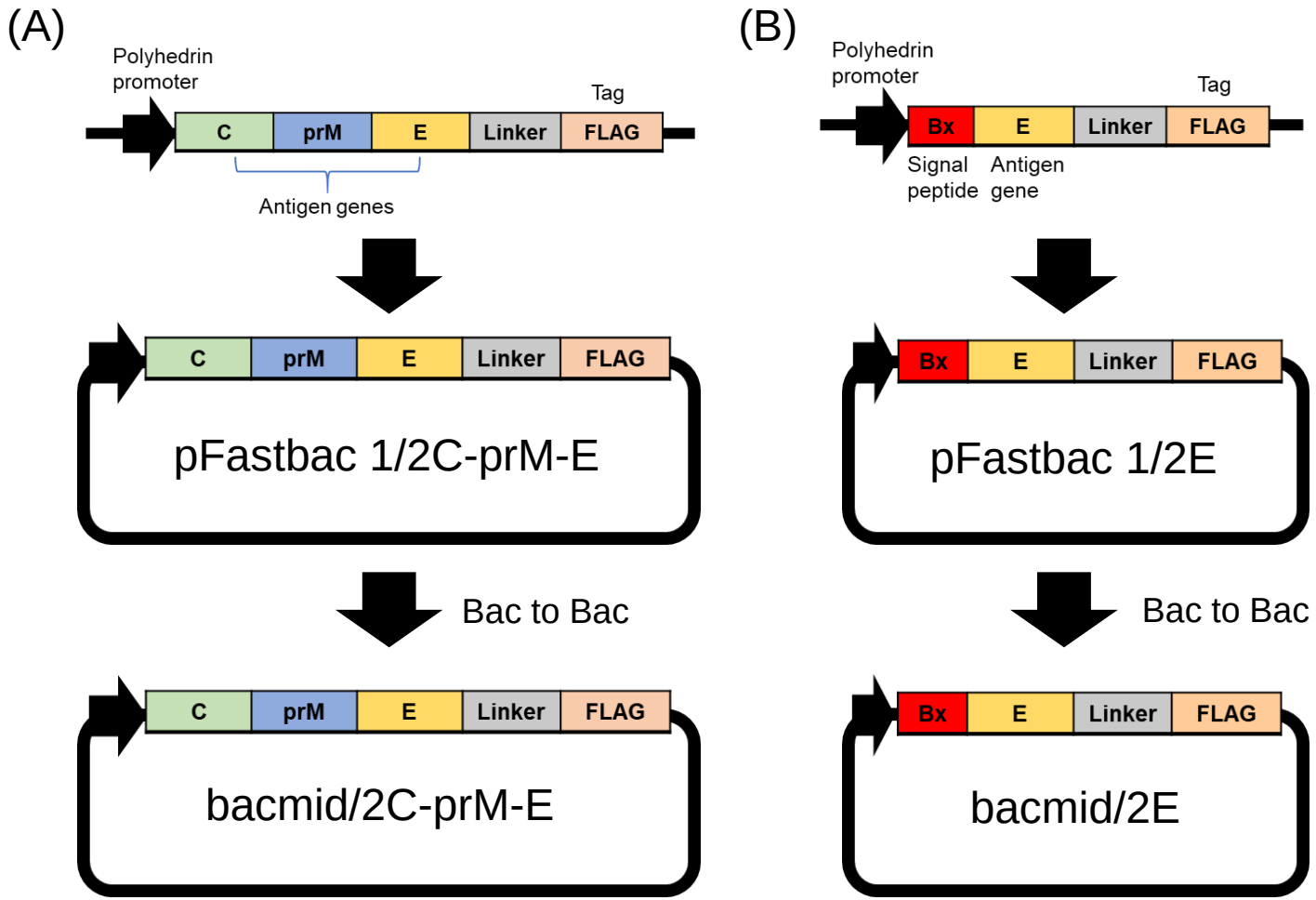
Fig. 2 Western blot of E protein and C-prM-E polypeptide expressed in silkworm larvae (A, C), pupae (B, D), and Bm5 cell (E). In the case of silkworm larvae hemolymph were collected after recombinant BmNPV infection. The homogenate of each sample was prepared according to the protocol described in Materials and methods. Expressed E protein was detected by western blot using a mouse anti-DDDDK tag antibody (A, B, E) or a rabbit anti-DENV E protein antibody (C, D), respectively, as a primary antibody.

Fig. 3 Western blot of purified C-prM-E polypeptide (A) and E protein (B), and their concentrated C-prM-E polypeptide (C) and E protein (D). Each protein was purified from silkworm hemolymph using DDDDK-tagged Protein PURIFICATION GEL column chromatography. Each eluate was concentrated by ultrafiltration and analyzed on SDS-PAGE (E).

Fig. 4 TEM analysis of purified C-prM-E polypeptide (A) and E protein (C). The purified C-prM-E polypeptide (B) and E protein (D) were immunogold labelled using anti-DENV2 E specific polyclonal antibody and analyzed on IEM.

Fig. 5 Binding assay of purified E protein (A) and C-prM-E polypeptide (B) to heparin

using ELISA. Heparin (1.8 ng) was coated onto each well of an ELISA plate and each amount of purified each protein was used for this ELISA which was carried out according to the protocol described in Materials and methods. Free heparin 1–4 μg was added to purified E protein (C) and C-prM-E polypeptide (D) for competitive ELISA assay.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Polyhedrin promoter
Tag
C
prM
E
Linker
FLAG
Antigen genes
C
prM
E
Linker
FLAG
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Bac to Bac
C
prM
E
Linker
FLAG
bacmid/2C-prM-E
(B)
Polyhedrin promoter
Tag
Bx
E
Linker
FLAG
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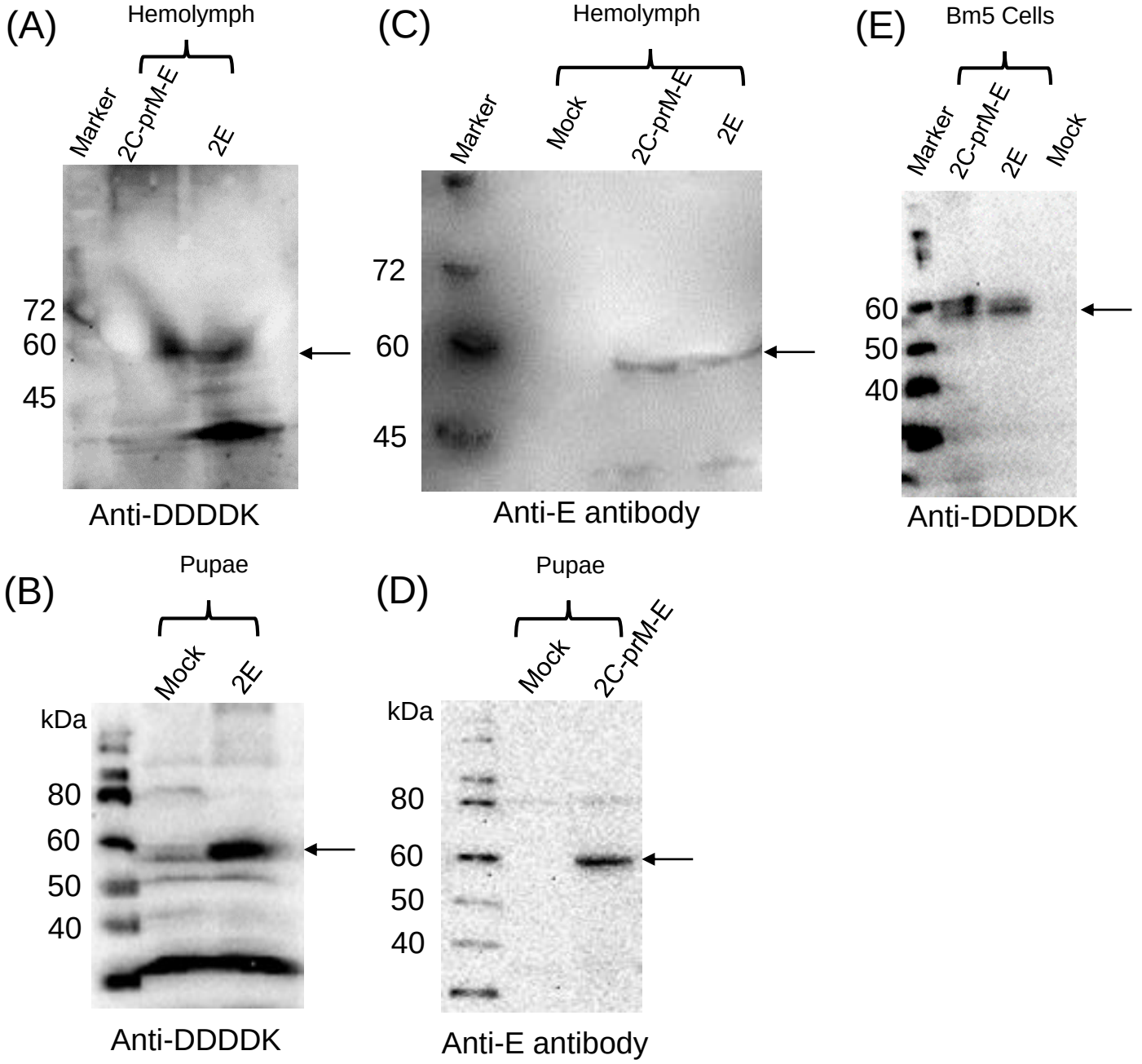
(A)
Hemolymph
Marker
2C-prM-E
2E
72
60
45
Anti-DDDDK
(C)
Hemolymph
Marker
Mock
2C-prM-E
2E
72
60
45
Anti-E antibody
(E)
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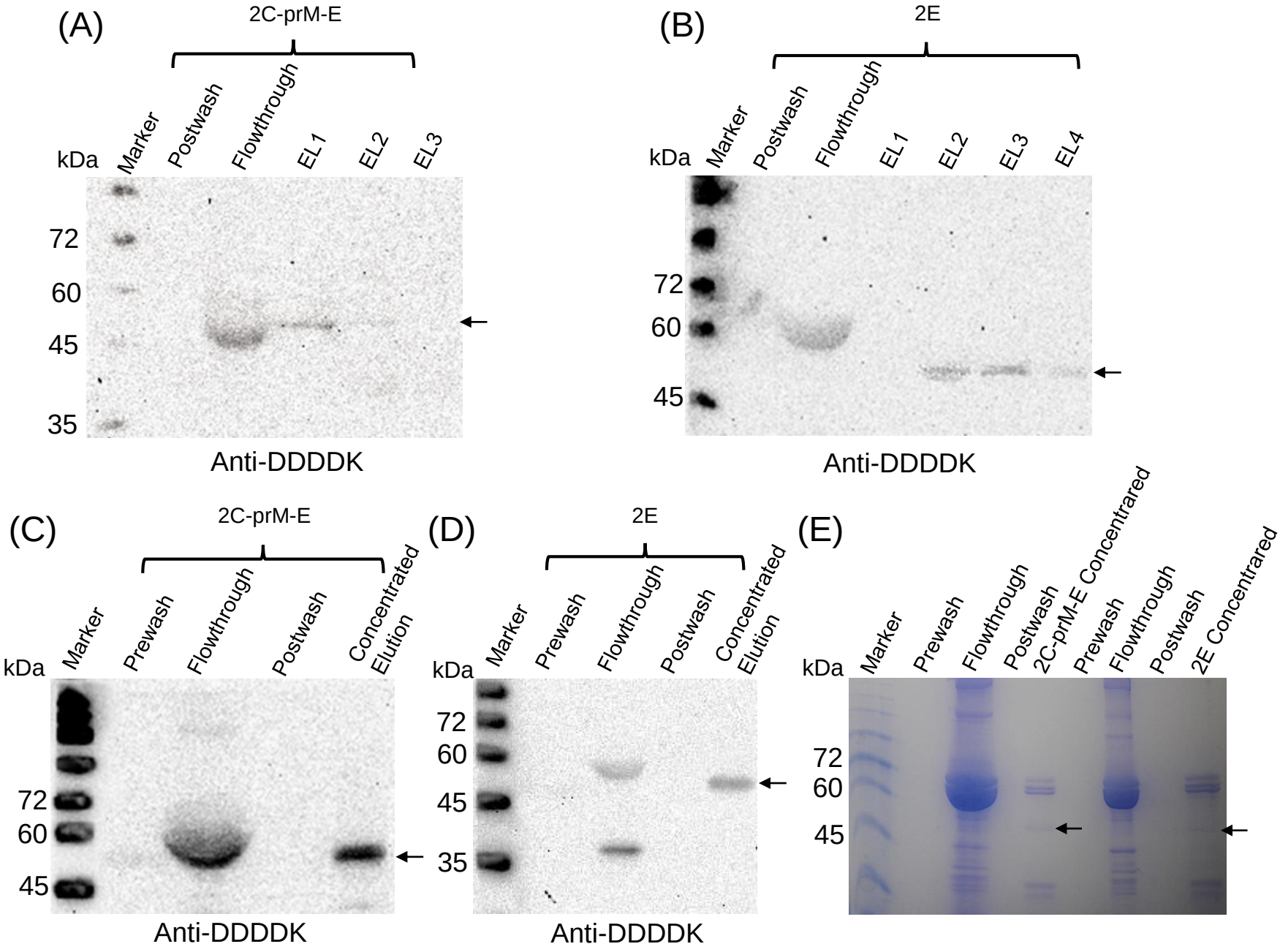
Anti-E antibody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2C-prM-E
Marker
Postwash
Flowthrough
EL1
EL2
EL3
kDa
72
60
45
35
Anti-DDDDK
(B)
2E
Marker
Postwash
Flowthrough
EL1
EL2
EL3
EL4
kDa
72
60
45
Anti-DDDDK
(C)
2C-prM-E
Marker
Prewash
Flowthrough
Postwash
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kDa
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Anti-DDDDK
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Marker
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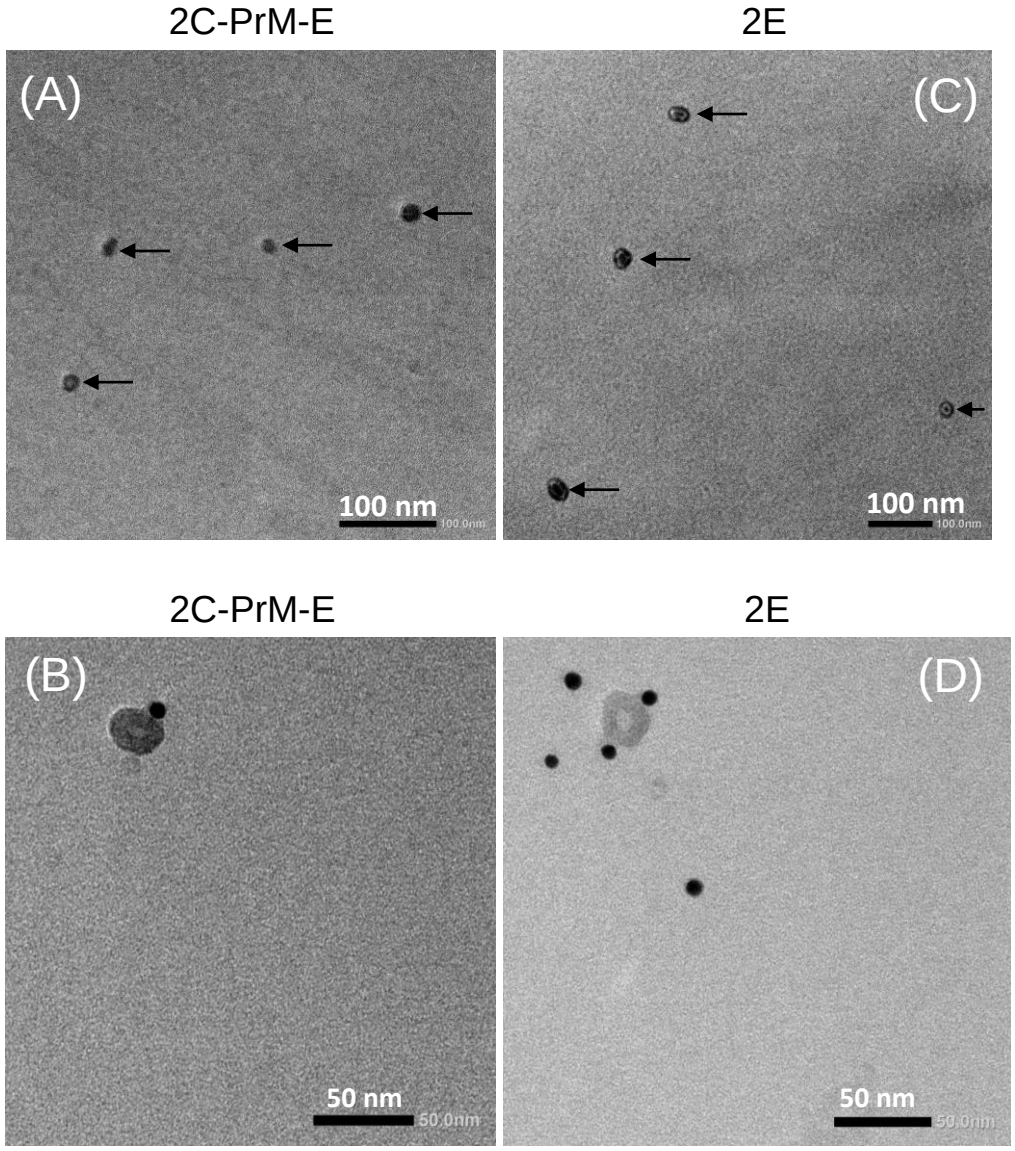
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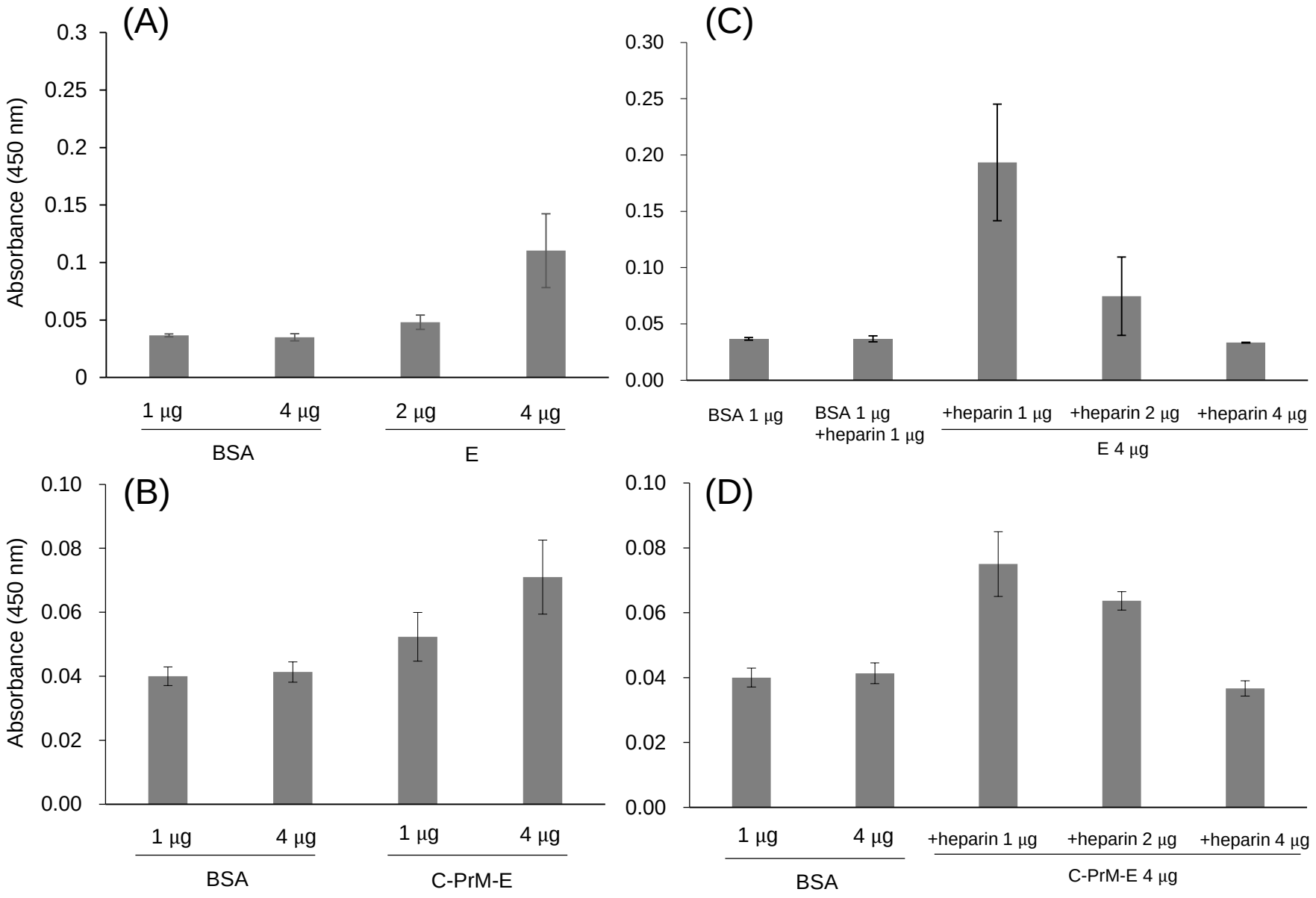
2E
(A)
(C)
100 nm
100 nm
2C-PrM-E
2E
(B)
(D)
50 nm
50 nm


(A)
Absorbance (450 nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
1 μg
4 μg
2 μg
4 μg
BSA
E
(C)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
BSA 1 μg
BSA 1 μg
+heparin 1 μg
+heparin 1 μg
+heparin 2 μg
+heparin 4 μg
E 4 μg
(B)
Absorbance (450 nm)
0.10
0.08
0.06
0.04
0.02
0.00
1 μg
4 μg
1 μg
4 μg
BSA
C-PrM-E
(D)
0.10
0.08
0.06
0.04
0.02
0.00
1 μg
4 μg
+heparin 1 μg
+heparin 2 μg
+heparin 4 μg
BSA
C-PrM-E 4 μg