Expression and purification of human (pro)renin receptor in insect cells using baculovirus expression system

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Abbreviations used: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; BES, baculovirus expression system; CBB, coomassie brilliant blue; CHAPS, 3-(3-cholamidepropyl)dimethylammonio-1-propanesulphonate; MOI, multiplicity of infection; hPRR, human (pro)renin receptor; hPRR-wTM, hPRR with transmembrane and C-terminal domain; hPRR-w/oTM, hPRR without transmembrane and C-terminal domain; ~~PIT~~d.p.i., days post-infection~~time~~; RA system, renin-angiotensin system

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Abstract

The renin-angiotensin (RA) system is important for the regulation of blood pressure and electrolyte balance, and renin is the rate-limiting enzyme in this system. The recent discovery of (pro)renin receptor (PRR) has reinforced the functional role of the RA system. PRR non-proteolytically activates prorenin and its role has attracted the attention of researchers towards the RA system. However, there is insufficient information on the biochemical structure and biological functioning of PRR due to the difficulty of measuring PRR expression. In this work, human PRR (hPRR) with intact transmembrane and C-terminal domain (hPRR-wTM) and PRR without this domain (hPRR-w/oTM) were expressed in insect cells using ~~BES~~baculovirus expression system (BES). Both hPRR-wTM and hPRR-w/oTM were fused with FLAG peptide by its N-terminus. Most of the hPRR-wTM was expressed in cell fraction~~intracellulary~~ and hPRR-w/oTM was secreted into the culture medium. hPRR-wTM was solubilized from the membrane fraction of recombinant baculovirus-infected cells by various detergents, suggesting that hPRR-wTM might be a transmembrane protein. hPRR-wTM was purified from the solubilized fraction using anti-FLAG M2 antibody agarose. Binding of purified hPRR-wTM to renin immobilized onto sensor chips was directly proportional to the hPRR-wTM concentration. Approximately ~~300~~225 μg of functional hPRR-wTM was purified from 80 ml of baculovirus-infected cell culture. Scale-up of this system will lead to mass production and crystallization of hPRR-wTM and determination of its biochemical structure and biological function.

Keywords: (pro)renin receptor, baculovirus expression system, purification, insect cells

Introduction

Various functional transmembrane and secretory proteins have been produced in insect cells by the baculovirus expression system (BES) and stably transformed cell system [1-4]. Insect cell expression systems are now commonly employed to produce functional mammalian proteins.

The renin-angiotensin (RA) system is a blood circulating system generating angiotensin II for regulating blood pressure and electrolyte balance. Prorenin is a precursor of renin, which is an important enzyme in the RA system. Prorenin is activated both proteolytically and non-proteolytically [5]. Recently, the non-proteolytic activation of prorenin bound to PRR was observed using recombinant rat prorenin and PRR [6]. The human (pro)renin receptor (hPRR) has been discovered and its gene cloned from a commercial human kidney expression library [7]. The hPRR consists of 350 amino acids with a signal peptide on the N-terminus and a single transmembrane domain near the C-terminus. PRR can bind to both renin and prorenin. It is assumed that the non-proteolytic activation of PRR-bound prorenin may be involved in the pathogenesis of hypertensive and diabetic tissue damage [8, 9]. (Pro)renin receptor blockade inhibits the progression of nephropathy and reverses the glomerulosclerosis that has already developed in diabetic rats [10, 11].

~~Moreover,~~ Suzuki et al. reported that the “gate and handle” regions of prorenin were crucial for its non-proteolytic activation [120~~]~~.~~,~~ ~~hypothesizing that the binding of PRR to the “handle” region of prorenin provokes the latter's activation.~~ However, an understanding of the mechanism by which PRR binds to prorenin is needed to further elucidate the pathophysiological roles of PRR. ~~However, the structure of hPRR and its~~

~~binding to (pro)renin have not been analysed..However, an understanding of the mechanism by which PRR binds to prorenin is needed to further elucidate the pathophysiological roles of PRR.~~

In this study, hPRR with (hPRR-wTM) or without (hPRR-w/oTM) the transmembrane and C-terminal domain was expressed in BES in order to obtain a large amount of hPRR and allowing the pathophysiological roles of PRR to be explored. hPRR-wTM was solubilized from the membrane fraction of recombinant baculovirus-infected insect cells and purified by anti-FLAG M2 antibody agarose. Binding of purified hPRR to human renin was confirmed using renin-immobilized sensor chips.

Materials and Methods

Construction of recombinant baculoviruses

hPRR-wTM was amplified by PCR with the primers prorenin F and prorenin R (Table 1). pENTER/D-GFP$_{uv}$-hPRR [13~~1~~] was used as a template. The amplified hPRR-wTM fragment contained a native putative signal sequence and FLAG sequence behind its signal sequence. Amplified hPRR-wTM was inserted into pENTR (Invitrogen, Carlsbad, CA, USA) by TOPO cloning, resulting in pENTR/hPRR-wTM being obtained. The hPRR-wTM gene was inserted into pDEST8 (Invitrogen) by Gateway Cloning Technology (Invitrogen) using pENTER/D-GFP$_{uv}$-hPRR, from which pDEST/hPRR-wTM was generated. The production of recombinant baculovirus containing the hPRR-wTM gene was performed according to the Bac-To-Bac

Expression System (Invitrogen) protocol. The recombinant baculovirus was named as AcMNPV/hPRR-wTM. hPRR without the transmembrane and C-terminal domain (hPRR-w/oTM) was amplified by PCR with prorenin F and prorenin delta R primers (Table 1). The recombinant baculovirus containing the human hPRR-w/oTM gene, AcMNPV/hPRR-w/oTM, was constructed by the same method as outlined above for AcMNPV/hPRR-wTM.

Culture of insect cells

Suspension cultures were generated in 100-ml flasks with a working volume of 20 ml medium, an agitation rate of 100 rpm and a temperature of 27^{o}C. Amplification of recombinant AcMNPV/hPRR-wTM and AcMNPV/hPRR-w/oTM was performed using Sf-9 cells (Invitrogen) with SF-900 II Serum Free Medium (Invitrogen). Amplified recombinant baculoviruses were collected at 2 days post-infection (d.p.i.)~~time (PIT)~~. For the expression of hPRR, *Trichoplusia ni* cells (Invitrogen) at a density of 2 x 10^{6} cells/ml were infected with recombinant baculovirus at a multiplicity of infection (M.O.I) of 5 and cultivated with Express Five Serum Free Medium (Invitrogen).

Cell viability and cell number were measured by 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO, USA) staining.

Solubilization and purification of hPRR-wTM and hPRR-w/oTM

T. ni cells infected with AcMNPV/hPRR-wTM were collected at 2 (d.p.i.)~~days PIT~~ and

suspended with 50 mM potassium phosphate buffer (pH 7.4, buffer A). The cells were disrupted by sonication using a ~~solicitor~~sonicator (VC 130PB, Sonic & Materials, Newtown, CT, USA). The homogenate was centrifuged at 8000 x g and the supernatant was subsequently centrifuged at 114,000 x g. The pellet was recovered and washed with buffer A before being suspended with 50 mM potassium phosphate buffer (pH 7.4) containing 150 mM sodium chloride (buffer B) and the suspension used as the membrane fraction.

To test the solubility of hPRR-wTM, Triton X-100, n-Dodecyl-β-D-maltoside, sodium cholate, CHAPS and n-Octyl-β-D-glucoside were used. ~~.~~ These detergents were first grade and purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). The membrane fraction was diluted to 4-5 mg of protein/ml and 1% (w/v) of the various detergents were added. The samples were incubated for 1 h at 4^{o}C before being centrifuged at 114,000 x g. The supernatants and pellets were analyzed by Western blot analysis.

The hPRR-wTM was purified from 80 ml of baculovirus-infected culture. The membrane fraction, collected as described above, was diluted with buffer B at 4-5 mg of protein/ml and 1% (w/v) Triton X-100 was added. The suspension was incubated over night and centrifuged at 114,000 x g. The supernatant was collected and used as ~~the~~ the solubilized membrane fraction. One ml of anti-FLAG M2 antibody agarose (Sigma) equilibrated with buffer B containing 0.05% Triton X-100 (buffer C) was added to the membrane fraction. The suspension of anti-FLAG M2 antibody agarose and the membrane fraction was stirred gently at 4^{o}C for 1 h, and the gel was then loaded into a column. The gel was washed with five volumes of buffer C. hPRR-wTM was eluted with 3 volumes of buffer C containing 100 μg/ml FLAG peptide (buffer D).

~~Putrification~~Purification of hPRR-w/oTM was performed similarly to that of hPRR-wTM using the culture supernatant at 2 d.p.i. and the buffer without the addition of Triton X-100. Eluted hPRR-wTM and hPRR-w/oTM was used in next ~~binding~~binding assay with Biacore biosensor.~~Eluted hPRR-wTM was used in the next binding assay with a Biacore biosensor.~~

Binding assay of hPRR-wTM to ligand using Biacore

For the study examining the binding of purified hPRR-wTM to renin by surface plasmon resonance, a Biacore biosensor (Biacore 2000; Biacore AB, Uppsala, Sweden) was used. The amine coupling method was used for the immobilization of purified recombinant human renin or ~~prorenin(~~prorenin (Cayman, Ann Arbor, MI, USA) onto a CM5 sensor chip. Unreacted carboxymethyl groups of a sensor chip lacking immobilized protein were blocked with ethanolamine as a control for non-specific binding. Various concentrations of purified hPRR solution were injected over the sensor chip. Buffer C was used as running buffer. Data analysis was performed with BIA evaluation ver. 3.1.

SDS-PAGE and Western blot analyses

SDS-PAGE was performed using the Mini-proteane II system (Bio-Rad, Hercules, CA, USA). Purified protein was visualized on the SDS-PAGE gel by staining with Coomassie Brilliant blue (CBB) R-250. After SDS-PAGE, proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot Electrophoretic

Transfer Cell (Bio-Rad). After blocking in 5% Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated in 1: 10,000 diluted anti-FLAG M2 antibody (Sigma) solution for 1 h. The membrane was washed and then incubated for 1 h in 1: 20,000 diluted anti-mouse IgG antibody labeled with horseradish peroxidase. Detection of hPRR was performed using ECL Plus (GE Healthcare, Buckinghamshire, UK) Western blotting reagent. Specific bands were detected using a Fluor-S/MAX multi-imager (Bio-Rad). Analysis of protein band of purified protein was performed by ~~Quantitiy~~Quantity One software (ver.4, Bio-Rad)

Results and discussion

Expression of hPRR-wTM and hPRR-w/oTM in T. ni cells

hPRR-wTM has a putative signal peptide on its N-terminus and one putative transmembrane domain near its C-terminus (Fig. 1). In previous work, the expression of hPRR-wTM fused with GFP_{uv} on its N-terminus was observed in the fat body of recombinant bacmid-injected silkworm larvae and the membrane fraction in its fat body cells [13~~1~~], suggesting that hPRR might be a transmembrane protein. In this work, hPRR-w/oTM was expressed in two forms in BES, hPRR-wTM– (pre-form: 40.0 kDa, mature-form: 38.5 kDa) which has the transmembrane and C-terminal domain and hPRR-w/oTM –(pre-form: 34.5 kDa, mature-form: 32.9 kDa) which lacks this domain.

T. ni cells were infected with either AcMNPV/hPRR-wTM or AcMNPV/hPRR-w/oTM at MOI 5 and cultivated for 5 days. hPRR-wTM was mainly

expressed ~~intracellularly~~ in cell fraction in AcMNPV/hPRR-wTM-infected cells at 2 and 3 ~~days PIT~~ d.p.i. and less so in the culture medium at 2 d.p.i.~~days PIT~~ (Fig. 2~~1~~). It assumed that hPRR-wTM might de displayed at the surface of baculovirus. It was reported that native forms of human receptors could be display at the surface of baculovirus even though without fusing with gp64 of baculovirus [14-16]. hPRR-wTM was not observed either ~~intra~~ in cell fraction or extracellularly at 5 d.p.i.~~days PIT~~ since expressed hPRR-wTM might be degraded by cysteine protease from AcMNPV [17~~12~~]. hPRR-w/oTM was principally expressed in the extracellular culture at 1-2 d.p.i.~~days PIT~~ (Fig. 2). Additionally, ~~intracellular~~ its expression in cell fraction was observed at 2-3 ~~days PIT~~d.p.i. (Fig. 2). However, at 3 d.p.i.~~days PIT~~, hPRR-w/oTM was observed only ~~intracellularly~~in cell fraction, suggesting that extracellular hPRR-w/oTM might be readily degraded by protease. This suggests that the extracellular protease activity was greater than that in the cell fraction~~intracellular culture~~ [17~~2~~].

Solubilization of hPRR-wTM in the membrane fraction of baculovirus-infected cells

The hPRR-wTM has one putative transmembrane domain, which may be expressed as a transmembrane protein. Since the hPRR-wTM was mainly expressed ~~intracellularly~~in the pellet of cell fraction, solubilization of hPRR-wTM was performed using the membrane fraction of AcMNPV/hPRR-wTM-infected cells (Fig. 3). hPRR-wTM was solubilized to the same extent by all detergents, indicating that the hPRR-wTM might be expressed as a transmembrane protein. The pellet fraction which was not solubilized by detergent contained degraded products, but these products were not detected in the solubilized fraction. In BES, the non-functional recombinant protein and its

aggregation were accumulated in the host's secretory pathway, suggesting that most of the hPRR-wTM in the pellet fraction which could not be solubilized by detergents was inactive.

Purification of hPRR-wTM and hPRR-w/oTM

Extracellulaly expressed hPRR-w/oTM was purified. Culture supernatant (80 ml) at 1-2 d.p.i.~~days PIT~~ (Fig. 2) was stirred gently after the addition of anti-FLAG M2 agarose gel, and then hPRR-w/oTM was eluted ~~–~~from the agarose gel with 100 μg/ml FLAG peptide. Finally 1.2 μg of hPRR-w/oTM was obtained. When 40 ng of the hPRR-w/oTM was loaded, N~~n~~o band was detected on the SDS-PAGE gel stained by CBB, but a faint band (approximately 33 kDa) was detected by Western blot (data not shown).

Conversely, the purified hPRR-wTM was confirmed by SDS-PAGE (Fig. 4). Purified hPRR-wTM at approximately 3~~7~~8 kDa (31-45 kDa) was identified by CBB staining with one minor band at around 66 kDa. ~~Apporoximately~~Approximately 75% of purified protein was occupied by hPRR-wTM. ~~–~~About ~~300~~ 225 μg of ~~partially purified~~ hPRR-wTM was obtained from 80 ml of cell culture medium. This means that the yield of hPRR-wTM was ~~250~~190-fold higher than that of hPRR-w/oTM.

Binding assay of hPRR-wTM to ligand by Biacore

The binding ability of hPRR-wTM to renin was tested by injecting various concentrations of purified hPRR-wTM (0-2.43 μM) over a purified recombinant

human renin-immobilized CM5 sensor chip (Fig. 5). A surface lacking immobilized human renin was used as a control. Binding of hPRR-wTM to human renin was directly proportional to the hPRR-wTM concentration. It was observed that hPRR-wTM bound to the chip depending on the concentration gradient, with the binding being enhanced at higher concentrations of hPRR-wTM. In the case of hPRR-w/oTM, when 60 nM of purified hPRR-w/oTM was applied over a human renin-immobilized CM5 sensor chip, binding of hPRR-w/oTM to renin could not be observed. It is likely that a very small amount of purified hPRR-w/oTM might cause no reaction with immobilized renin. Whether the purified hPRR-w/oTM can bind with renin or prorenin should be investigated in the near future.

Binding of hPRR-wTM to purified recombinant human prorenin immobilized onto a CM5 chip using the same method could not be observed (data not shown). This may be because the site at which prorenin binds to hPRR-wTM might be hidden by the immobilization of human prorenin onto a CM5 chip.

Summary

The expression and partial purification of functional human hPRR-wTM in insect cells was achieved in this study. Although hPRR was discovered and its gene was cloned [7], purified hPRR was not available for research. Prorenin bound to PRR was activated non-proteolytically [6] and its binding was associated with the activation of MAP kinases, ERK1 and ERK2 [7]. Analysis of the structure of PRR assists with understanding the mechanism by which the RA system operates. In volume, approximately 2.8~~3.75~~ mg of the purified hPRR-wTM can be obtained from 1 liter of

baculovirus-infected cell culture using this system. It has been reported that a larger amount of transmembrane and secretory proteins can be expressed in a stably transformed cell system than in BES [3, 183]. It is expected that the crystallization of hPRR-wTM can be achieved by an improvement in the genetic construction of cell culture, providing an enhanced purified yield.

Acknowledgements

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Table. 1 Primers used

Name	5' - 3'
Prorenin F	CACCATGGCTGTGTTTGTCGTGCTCCTGGCGTTGGTG
	GCGGGTGTTTTGGGGGACTACAAGGACGACGACGACAAG
Prorenin R	ACGGAATTCTAATCCATTCGAATCTTCTGG
Prorenin delta R	AACTGCAGCTGACTACAAGGACGACGACGAC

Figure legends

Fig. 1. (A) Amino acid sequence of hPRR. Arrow indicates the point of truncation. (B) Constructions of ~~ed~~ hPRR-w/oTM and hPRR-wTM.

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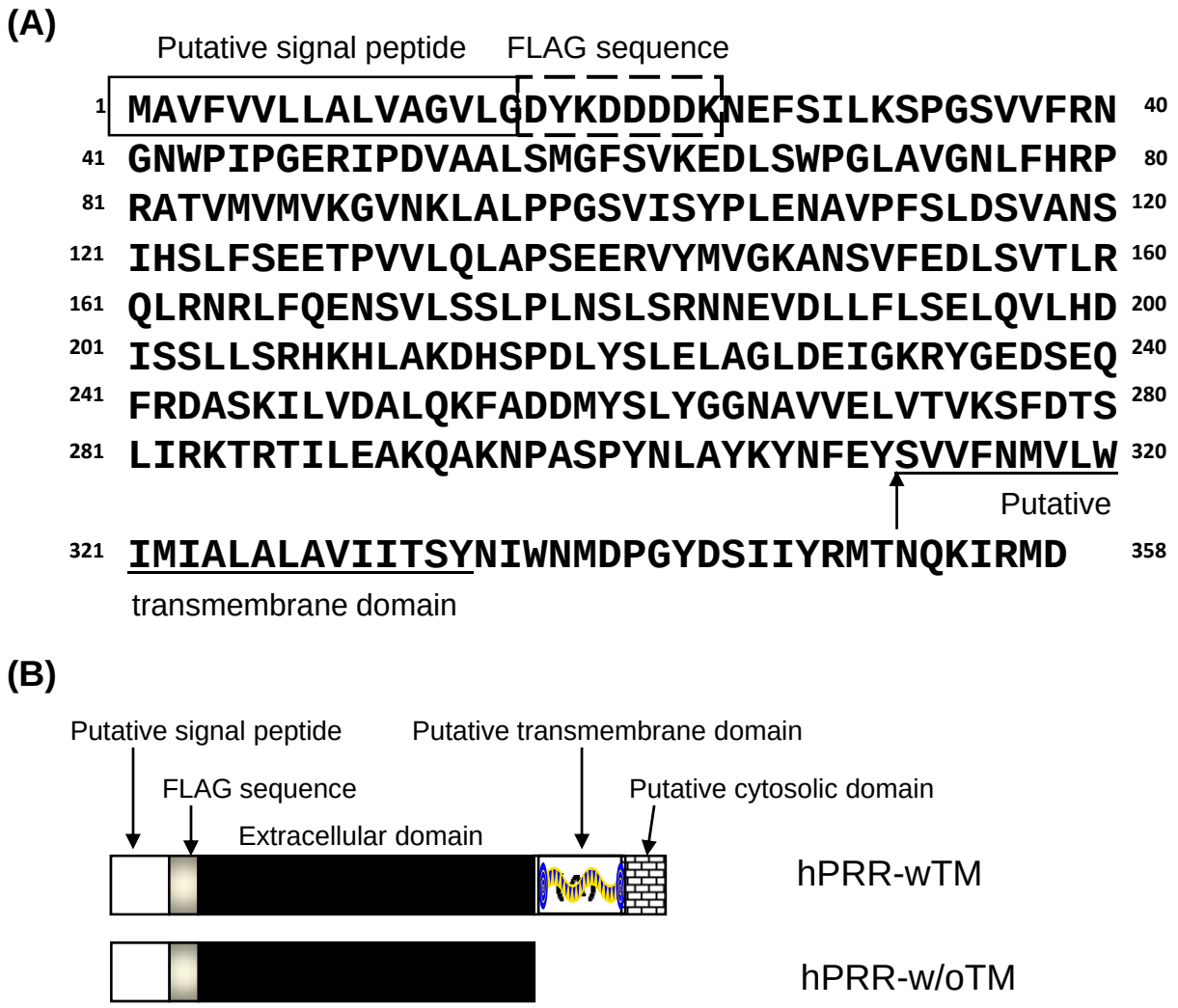
Fig. 2~~1~~. Western blot of hPRR-wTM (A) and hPRR-w/oTM (B) expressed in cell fraction~~tra~~ and extracellularly in *T. ni* cells. One hundred ml of each cell culture was centrifuged and the supernatant separated from infected *T. ni* cells was used as extracellular samples. Cells were suspended by 100 ml of phosphate buffer (pH 7.4) containing 1% (w/v) Triton X-100 and the suspension was diluted 100-fold with the same buffer. The diluted suspension was used as the ~~intracellular~~cell fraction samples. Lanes 1-5 denote d.p.i.~~PIT~~ (d) and lane M denotes the molecular weight marker. Arrows in (A) and (B) indicate hPRR-wTM and hPRR-w/oTM, respectively.

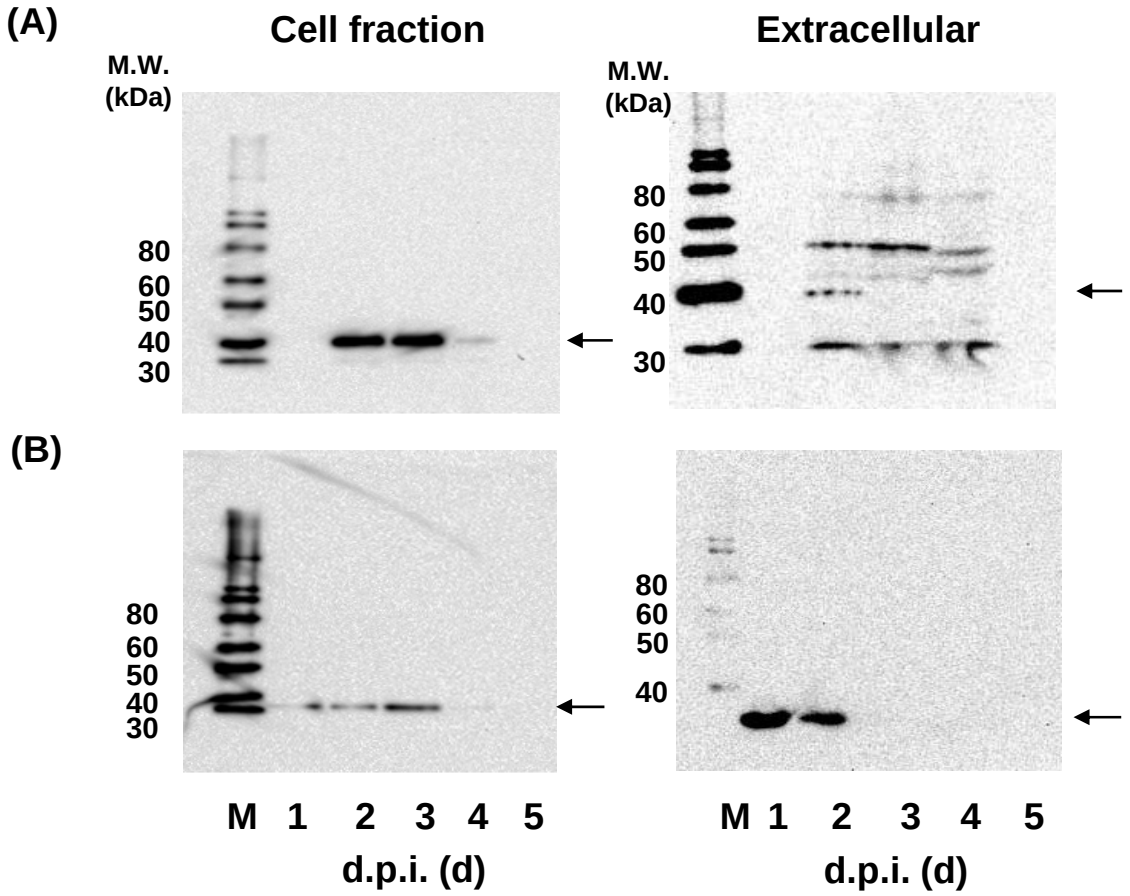
Fig. 3~~2~~. (A) Solubilization of hPRR-wTM from the membrane fraction of AcMNPV/hPRR-wTM-infected *T. ni* cells by various detergents~~.~~. The solubilization method is described in the Materials and Methods. Lanes; M: molecular weight marker, 1: Triton X-100, 2: n-Dodecyl-β-D-maltoside, 3: Sodium cholate, 4: CHAPS, 5: n-Octyl-β-D-glucoside. Arrow indicates hPRR-wTM. (B) ~~Tn :~~ Solubilized membrane fraction of *T. ni* cells (Tn) without recombinant baculovirus infection. ~~Arrow indicates hPRR-wTM.~~

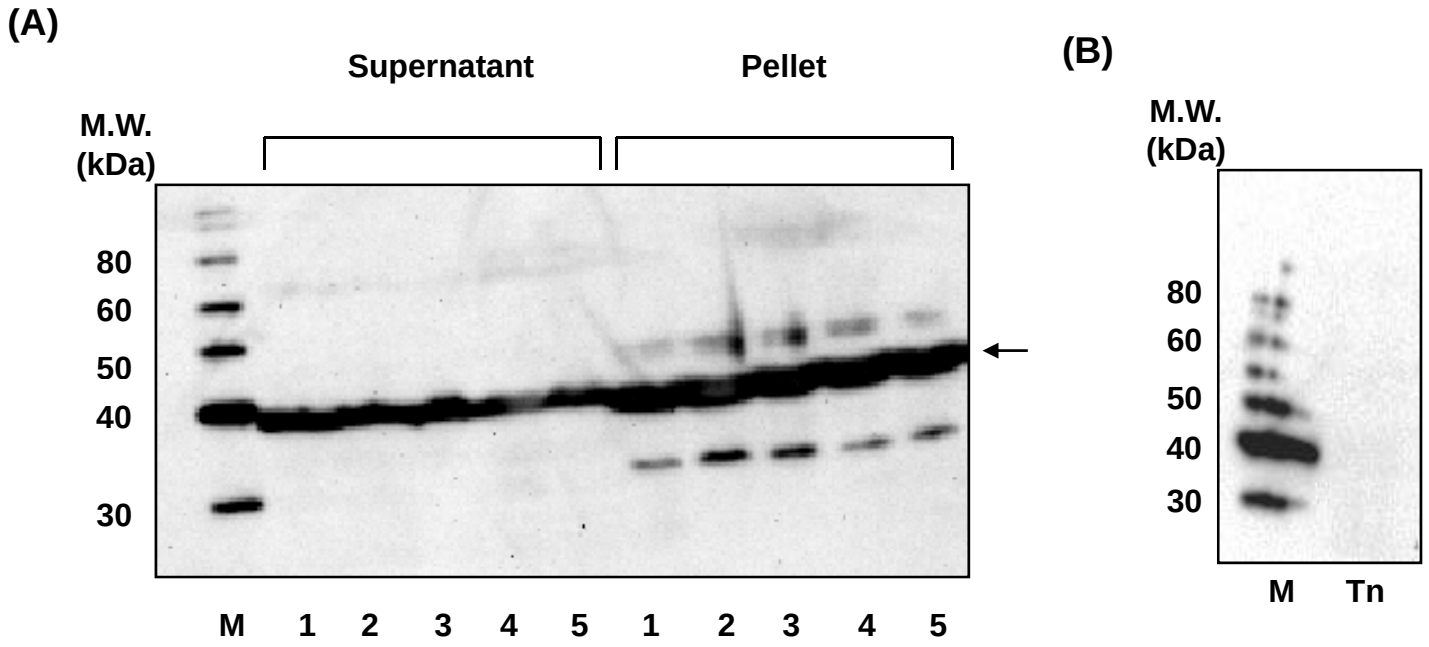
Fig. 4~~3~~. SDS-PAGE analysis of purified hPRR-wTM. 10% polyacrylamide gel was used and stained by Coomassie Brilliant Blue (CBB) R-250. Lanes; 1: Solubilized

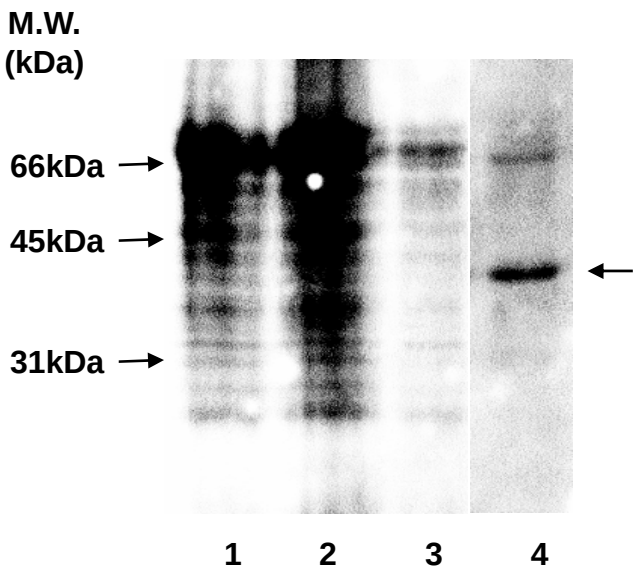
fraction, 2: anti-FLAG M2 antibody flow-through fraction, 3: anti-FLAG M2 antibody washed fraction, 4: anti-FLAG M2 antibody eluted fraction. Arrow indicates hPRR-wTM.

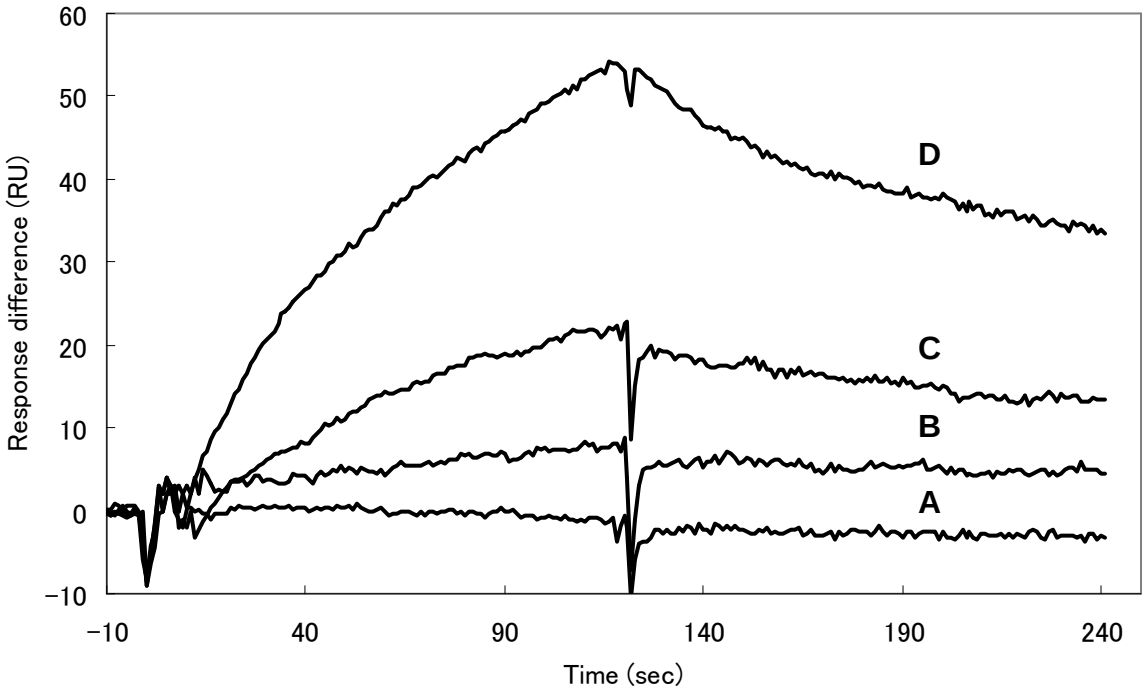
Fig. 54. Biacore analysis of the binding of hPRR-wTM to human renin immobilized onto a CM5 chip. Sensorgram overlays of various concentrations of hPRR injected over immobilized human renin are shown. Unreacted carboxymethyl groups of a sensor chip lacking immobilized protein were blocked with ethanolamine as a control for non-specific binding. The Biacore procedure is described in the Materials and methods. A, B, ~~and~~ C, and D denote partial purified hPRR-wTM concentrations of 0, 0.1249, 0.97 and 2.43 μM, respectively.


(A)
Putative signal peptide
FLAG sequence
1 MAVFVVLLALVAGVLGDYKDDDDKNEFSILKSPGSVVFRN 40
41 GNWPIPGERIPDVAALSMGFSVKEDLSWPGLAVGNLFHRP 80
81 RATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANS 120
121 IHSLFSEETPVVLQLAPSEERVYMVGKANSVFEDLSVTLR 160
161 QLRNRLFQENSVLSSLPLNSLSRNNEVDLLFLSELQVLHD 200
201 ISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQ 240
241 FRDASKILVDALQKFADDMYSLYGGNAVVELVTVKSFDTS 280
281 LIRKTRTILEAKQAKNPASPYNLAYKYNFEYSVVFNMVLW 320
Putative
321 IMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 358
transmembrane domain
(B)
Putative signal peptide
Putative transmembrane domain
FLAG sequence
Putative cytosolic domain
Extracellular domain
hPRR-wTM
hPRR-w/oTM


(A)
Cell fraction
Extracellular
M.W.
(kDa)
80
60
50
40
30
(B)
M 1 2 3 4 5
d.p.i. (d)


(A)
Supernatant
Pellet
M.W.
(kDa)
80
60
50
40
30
M 1 2 3 4 5 1 2 3 4 5
(B)
M.W.
(kDa)
80
60
50
40
30
M Tn


M.W.
(kDa)
66kDa
45kDa
31kDa
1
2
3
4


60
50
40
30
20
10
0
−10
Response difference (RU)
D
C
B
A
−10
40
90
140
190
240
Time (sec)