Binding Affinity of Full−length and Extracellular Domains of Recombinant Human (Pro)renin Receptors to Human Renin When Expressed in the Fat Body and Hemolymph of Silkworm Larvae

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Abstract

Transmembrane domains of some receptors have been found to be very important in the process of constitutive oligomerization, and in the stability and functioning of the receptor. The human (pro)renin receptor (hPRR) is composed of an extracellular domain, a transmembrane domain and a cytoplasmic domain, which binds to both renin and prorenin. In this study, full−length of hPRR (hPRR) and hPRR lacking cytoplasmic domain (hPRR−ΔCD) were found to be expressed in fat body of silkworm larvae, and the extracellular domain of hPRR (hPRR−ΔTMΔCD) in hemolymph. Three forms of hPRR were investigated the mechanism of interaction between receptor and ligand using real−time monitored surface plasmon resonance (SPR) equipped with immobilized human renin onto one cuvette channel. As a result, the transmembrane domain of hPRR is indispensable in the formation of functional hPRR. The dissociation equilibrium constants (K_D) of purified hPRR and hPRR−ΔCD were estimated to be 46 nM and 330 nM, respectively. No evidence of binding by hPRR−ΔTMΔCD located in hemolymph was found either before or after purification. To our knowledge, these are the first findings describing the interaction of transmembrane and extracellular domains of hPRR with ligand and this may help towards the understanding of binding affinity of hPRR to ligand.

(**Key words:** human (pro)renin receptor; binding affinity; surface plasmon resonance; silkworm larvae)

INTRODUCTION

Human (pro)renin receptor (hPRR) is composed of an extracellular domain, a transmembrane domain and a cytoplasmic domain. Following cloning from a human kidney cDNA library, hPRR was found to bind to both renin and prorenin (1). Suzuki *et al*. showed that specific protein binding with the “handle” region of the prosegment in human prorenin led to its non-proteolytic activation presumably by conformational changes, and predicted that such a region played a key role in the binding with specific proteins (2). Recently, a large number of studies have reported that this receptor, and its mechanism of binding to renin/prorenin, involves the generation or action of angiotensin, leading to numerous cardiovascular diseases (3−5). Consequently, the development of hPRR receptor blockers is receiving considerable attention at present. Also, an understanding of the functional properties of hPRR through detailed biochemical and biophysical analysis is urgently required.

Many reports examining receptor functionality and structure have found the extracellular domain to play a key role in affecting the binding affinity with the receptor's ligand (6, 7). Given this, expression of the typically soluble extracellular domain of a receptor, rather than its full−length form, can be employed to study the functions of a protein. Through this approach, the use of detergents can be avoided and research on protein structure and function would be facilitated. In contrast, the transmembrane domains of some receptors have been found to be very important in the process of constitutive oligomerization (8), and in the stability (9) and functioning of

the receptor (10). If this is the case, preparation of the full−length of protein will be necessary. However, to our knowledge, the direct binding affinity of full−length hPRR and its extracellular domain to ligand has not been reported until now.

In a previous study, we successfully expressed full−length hPRR in silkworm larvae, and identified its location in the microsomal fraction of the fat body (11). In this study, the full−length hPRR (hPRR), hPRR lacking cytoplasmic domain (hPRR−ΔCD), and extracellular domain of hPRR (hPRR−ΔTMΔCD) were expressed in silkworm larvae to investigate the mechanism of interaction between receptor and ligand using real−time monitored surface plasmon resonance (SPR).

MATERIALS AND METHODS

Construction of recombinant BmMNPV/hPRR, /hPRR−ΔCD, /hPRR−ΔTMΔCD, and /ΔBmgp64−hPRR−ΔTMΔCD bacmids The full−length human PRR gene and recombinant BmMNPV/hPRR bacmid were prepared as previously described (11). In a similar manner, the hPRR gene lacking the transmembrane and cytoplasmic domain (hPRR−ΔTMΔCD) was amplified for secretory expression by PCR with the concurrent introduction of cloning sites *Pst* I and *Eco*R I at the 5' and 3' terminus, respectively, and the introduction of a FLAG tag behind the *Pst* I site. The 5'primer sequence (F1) was TCACTGCAGACTACAAGGACGACGACGACAAGAACGAGTTTAGTATATTAA AATCACCAG whilst that of the 3'primer (R1) was

GACGAATTCCTAATATTCAAAATT (cloning sites are underlined, the FLAG codons are fenced). The stop codon was introduced immediately following the last extracellular domain codon (Tyr311) and before the *Eco*RI site to prevent further translation. The amplified hPRR−ΔTMΔCD digested by *Pst* I and *Eco*R I was cloned into pBlueBacHis2/GFP_{uv} which was also digested by *Pst* I and *Eco*R I (12). For addition of the foreign bombyxin signal sequence, the resulting construct was amplified using 5'−CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTG GGTGTCAACACAACCGCGGGGTTCTCATCATC−3' as the 5' primer (F2) and the 3' primer (R1) described above. The PCR product was inserted into pENTR (Invitrogen, Carlsbad, CA, USA) by TOPO cloning. After verification of the DNA sequence, the resulting pENTR/D−hPRR−ΔTMΔCD was transferred into pDEST 8 by Gateway Cloning Technology (Invitrogen). The pDEST8/hPRR−ΔTMΔCD plasmid generated was transformed into *E. coli* Bm DH10Bac competent cells containing the BmMNPV bacmid (13) and the white Kanamycin and Gentamicin−resistant colonies were selected. The recombinant BmMNPV/hPRR−ΔTMΔCD bacmid was isolated and its identity confirmed by PCR using the 5' primer described above and M13 reverse sequencing primer (Invitrogen).

The human PRR gene lacking the cytoplasmic domain (hPRR−ΔCD) was amplified from the pENTR/D−hPRR (11) by PCR with the 5'primer (F2) as described above and 5'−TCAGTAAGAGGTGATAATCACAGCCAAGGCCAAGGCGATC−3' as the 3'primer (R2). The PCR product was inserted into pENTR by TOPO cloning,

and the following procedures were performed in the same construction of BmMNPV/hPRR−ΔTMΔCD bacmid as described above.

BmMNPV bacmid containing ΔBmgp64−hPRR−ΔTMΔCD fusion gene was constructed as follows. The gp64 gene from BmMNPV was amplified by PCR using the Bmgp64F primer 5'−CACCGGTACCATGGTAGGCGCTAATGTTTTATACG−3' and Bmgp64R primer 5'−CCCAAGCTTTTAATATTGTCTACTATTACGGTTTC−3'. The amplified fragment was cut by *Kpn* I and *Hind* III, and inserted into pUC18 digested by *Kpn* I and *Hind* III. The plasmid thus constructed was named pUC−Bmgp64. The hPRR−ΔTMΔCD gene was amplified by PCR using BGL−hPRR−F 5'−GAAGATCTCGACTACAAGGACGACGACGACAAAG−3' and SPH−hPRR−R 5'−ACATGCATGCATATATTCAAAATTATACTTATATGC−3' and digested by *Bgl* I and *Sph* I. The digested hPRR gene was inserted into the *Bam*H I−*Sph* I site of the gp64 gene in pUC−Bmgp64 to yield phPRR−ΔBmgp64. The ΔBmgp64−hPRR−ΔTMΔCD fusion gene was amplified by PCR using Bmgp64F and Bmgp64R, and inserted into a pENTR/D/TOPO vector by TOPO cloning. The procedures which followed after this process were performed using the same protocol as for the construction of the recombinant BmMNPV/hPRR−ΔTMΔCD bacmid.

Injection of recombinant BmMNPV bacmids into silkworm larvae

Fifth−instar silkworm larvae (Ehime Sansyu, Ehime, Japan) were injected with the recombinant BmMNPV bacmid DNA solutions (4 μg of bacmid DNA, 5 μl of DMRIE−C transfection reagent (Invitrogen), dissolved in PBS buffer to 45 μl per larva). Five days post−injection, the hemolymph and fat body were collected by cutting

a caudal leg and dissection, respectively.

Preparation of crude samples The fat body was sonicated in homogenization buffer comprising 50 mM sodium phosphate, 150 mM NaCl and protease inhibitors (Roche, Lewes, Sussex, UK) (pH 7.4; Buffer A). Unsonicated cells and cellular debris were removed by centrifugation at 600 × g for 10 min followed by a second centrifugation of the supernatant at 8000 × g for 30 min. The microsomal fraction was collected from the resulting supernatant by centrifugation at 114,000 × g for 60 min and was washed twice with buffer A. To solubilize hPRR from the microsomal fraction, a solubility test using various detergents was performed (see supplementary file 1). The pelleted membrane fraction was resuspended in extraction buffer (50 mM sodium phosphate, 150 mM NaCl, protease inhibitors and 1.5% dodecyl−β−D−maltoside (DDM), pH 8.0; Buffer B) to a final concentration of 5 mg/ml. Solubilized membrane proteins were recovered by collection of the supernatant after centrifugation at 114,000 × g for 60 min.

Hemolymph was pre−treated by a 10−fold dilution with Buffer A and centrifugation at 114,000 × g for 60 min to allow the complete removal of insoluble materials.

Every procedure described above was carried out at 4°C whilst the prepared samples were stored at −80°C for use in future binding assays or for purification.

Purification of hPRRs The soluble microsomal fraction of the fat body was incubated in batches with 0.5 ml of anti−FLAG M2 antibody agarose resin (Sigma−Aldrich, St. Louis, MO, USA) for 1.5 hours at 4°C. And then the resin was loaded into

a gravity column and washed with 10 ml of wash buffer (50 mM sodium phosphate, 150 mM NaCl, protease inhibitors and 0.2% DDM, pH 8.0; Buffer C). The hPRR was eluted with 4.5 ml of elution buffer (50 mM sodium phosphate, 150 mM NaCl, 0.2% DDM, 100 μg/ml of FLAG peptide and protease inhibitors, pH 8.0; Buffer D).

Purification from pre−treated hemolymph was performed by following a similar procedure using anti−FLAG M2 antibody agarose resin, but without the addition of detergent to the buffers.

The protein concentration was measured by using a BCA protein assay reagent kit (Thermo Scientific Rockford, IL, USA) and bovine serum albumin as a standard.

SDS−PAGE and Western blotting analysis SDS−PAGE was performed in 10% polyacrylamide gel in a Mini−protean II system (Bio−Rad, Hercules, CA, USA). The GFP_{uv}−fused protein was visualized using a Molecular−FX multi−imager (Bio−Rad) and/or by stained with Coomassie Brilliant Blue (CBB) R−250. Western blotting was performed by using anti−FLAG−M2 antibody (10,000−fold dilution, Sigma) as the primary antibody and anti−mouse IgG antibody conjugated to horseradish peroxidase (20,000−fold dilution; GE Healthcare, Buckinghamshire, UK) as the secondary antibody. The immunoreactive proteins were visualized by ECL plus Western blotting detection reagents (GE Healthcare) and detected using a Fluor−S/MAX multi−imager (Bio−Rad).

Surface plasmon resonance assays Real−time monitored SPR assays were performed at 25°C using an IAsys plus biosensor (Labsystems Affinity Sensors, Cambridge, UK). The instrument employs a dual−well stirred cuvette (Labsystems

Affinity Sensors) precoated with carboxymethyl dextran (CMD). The amine coupling method was used for the immobilization of purified recombinant human renin (Cayman, ANN Arbor, MI, USA) onto one cuvette channel (C1). The other cuvette channel (C2) was used as a control for non−specific binding. Unreacted carboxymethyl groups of the sensor cuvette lacking immobilized protein were blocked with ethanolamine. The chip surface was regenerated by adding 10 mM HCl until the response signal returned to base line. The experimental data were processed using the IAsys FASTfit software. The association rate constant k_{ass} was obtained as a slope of the measured k_{on} values plotted against the protein concentration used in this experiment. The dissociation rate constant k_{diss} was determined from the dissociation experiment. The dissociation equilibrium constant (K_D) was defined as: $K_D=k_{diss}/k_{ass}$.

RESULTS AND DISCUSSION

Expression and purification of three forms of hPRRs The hPRR and hPRR−ΔTMΔCD fusion proteins (Fig. 1A) collected from the hemolymph of silkworm larvae were analyzed by Western blotting using anti−FLAG−M2 antibody (Fig. 1B). Compared with the weak band detected for hPRR in the hemolymph, hPRR−ΔTMΔCD lacking its transmembrane and cytoplasmic domains showed a single strong band on during Western blotting, indicating that it was secreted extracellularly. Additionally, hPRR−ΔTMΔCD showed the expected molecular weight of 64 kDa. No band was

detected in the mock−injected silkworm larvae.

The hPRR and hPRR−ΔCD fusion proteins were mostly expressed in fat body as shown in their GFP_{uv} fluorescence analysis (Fig. 1C), but were not detected in the hemolymph of silkworm larvae (data not shown). The size of the hPRR and hPRR−ΔCD was estimated to be 69 and 65 kDa, respectively, which was found to be principally located in the microsomal fraction of the fat body (11).

The hPRR, hPRR−ΔCD, and hPRR−ΔTMΔCD fusion proteins expressed in the fat body were purified by simple one−step anti−FLAG affinity chromatography. The results of SDS−PAGE and GFP_{uv} fluorescence analysis of purified hPRR, hPRR−ΔCD, and hPRR−ΔTMΔCD are shown in Fig. 2A. For hPPR and hPRR−ΔCD, a major band of 69 and 65 kDa was observed. In the case of hPRR−ΔTMΔCD, SDS−PAGE showed the predicted band of 64 kDa, whereas some degraded bands below the main band were also detected by the GFP_{uv} fluorescence analysis. This suggests that the lack of a transmembrane domain may render hPRR unstable and be readily degraded compared with the full−length version during the same purification procedure. In addition, hPRR−ΔTMΔCD could not be purified with the insect cell expression system (12) either because of this extracellular instability or because of its low expression level.

Binding assay for crude and purified hPRRs In order to compare and characterize the binding affinities of hPRR and hPRR−ΔTMΔCD, SPR experiments were carried out using an IAsys plus biosensor. Four hundred arc seconds of human renin were coupled on the cuvette of the IAsys plus biosensor. According to the method described by Li *et al* (14), the amount of immobilized renin was calculated as

2.45 pmol. The prepared soluble microsomal fraction of fat body expressed hPRR and the hemolymph expressed hPRR−ΔTMΔCD were diluted to 3 mg/ml and injected into the renin immobilized cuvette. The same treatments were also performed using the microsomal fraction and hemolymph of mock−injected silkworm larvae as a control. The results derived from the real−time response curve showed the binding of hPRR from the fat body to be three times higher than that of the mock controls at the point of maximum response. The difference detected was 84 arc seconds (Fig. 2B). It can be calculated that 1 μg of crude protein containing hPRR increased the response by 0.56 arc seconds. However, no difference was observed between hPRR−ΔTMΔCD obtained from the hemolymph and the mock controls (Fig. 2B). The hPRR−ΔTMΔCD was expressed partly in hemolymph (Fig. 1B) and partly in fat body. Binding affinity showed only in the microsomal fraction of fat body. The hPRR−ΔTMΔCD is composed of hydrophilic and hydrophobic parts that might be associated with membrane, which might be the reason of showing binding affinity.

To further determine the binding affinities of hPRR−ΔTMΔCD, purified hPRR and hPRR−ΔTMΔCD were diluted to the same concentration in 50 mM sodium phosphate buffer containing 150 mM NaCl and 0.2% DDM, and then analyzed using the biosensor. The binding affinity of hPRR was confirmed whilst hPRR−ΔTMΔCD was found to show no such affinity (Fig. 2C). This suggests that full−length hPRR, but not the secreted extracellular domain, possesses the ability to bind with human renin.

Binding assays using different concentrations (0.13~1 μM) of purified hPRR and hPRR−ΔCD were also performed. The response of hPRR−bound renin showed a

typical concentration−dependent distribution as shown in Fig. 2D. The specific binding responses at various hPRR concentrations (0.13, 0.25, 0.5, 1μM) were calculated as 5.1, 5.5, 6.8 and 9.1 arc seconds/μg, respectively; values which were almost 10 times higher than that of crude protein. The dissociation equilibrium constant (K_D) was estimated by the IAsys FASTfit software to be 46 nM (see supplementary file 2). To verify the action of cytoplasmic domain of PRR, the binding assay of purified hPRR−ΔCD was performed at various concentrations (0.13-1μM). Also, this purified one showed binding affinity (Fig. 2D). The K_D was estimated to be 330 nM.

The K_D of hPRR when bound to prorenin has previously been reported as 6.6 nM when measured using an ELISA method (12). In another study, the K_D values for rat PRR binding rat prorenin and renin were 8.3 nM and 20 nM, respectively (15). The range of binding affinities obtained from rats is similar to that of humans, probably due to an amino acid sequence homology of greater than 83% between the receptors of the two species.

Binding assay for hPRR−ΔTMΔCD fused with BmMNPV gp64 protein

To further investigate whether the binding properties of hPRR were affected by the native transmembrane domain, ΔBmgp64−hPRR−ΔTMΔCD consisting of three domains; Bmgp64 *N*−terminal domain (1−82 amino acids), hPRR−ΔTMΔCD and a Bmgp64 *C*−terminal domain (321−511 amino acids) (Fig. 3A), was constructed. Since the ΔBmgp64−hPRR−ΔTMΔCD has a heterologous transmembrane domain, it was expressed in the fat body, as confirmed by Western blotting (Fig. 3B). The ΔBmgp64−hPRR−ΔTMΔCD in the microsomal fraction showed a binding affinity

(0.51 arc seconds/μg crude protein) (Fig. 3C) but the purified one did not (Fig. 3D). It is inferred that the heterologous transmembrane domain cannot be replaced by a native transmembrane domain from hPRR.

Binding assay for hPRR−ΔTMΔCD expressed in the fat body To make clear why the binding affinity of ΔBmgp64−hPRR−ΔTMΔCD in the microsomal fraction was lost by its purification, hPRR−ΔTMΔCD was expressed in the fat body. The microsomal and soluble fractions of the fat body were extracted from BmMNPV/hPRR−ΔTMΔCD bacmid−injected silkworm larvae as described in the Materials and Method section. The amount of hPRR−ΔTMΔCD in the microsomal fraction was smaller than that of soluble fraction (Fig. 4A). Interestingly, however, its binding affinity was similar to that of hPRR (Fig. 4B; see Fig. 2B). On the other hand, the hPRR−ΔTMΔCD in the soluble fraction showed a similar binding affinity to the corresponding fraction of the mock control samples (Fig. 4B). The specific response of hPRR−ΔTMΔCD in the microsomal fraction was 0.48 arc seconds/μg crude protein. Unexpectedly, the purified hPPR−ΔTMΔCD from the microsomal fraction was not found to bind with renin (Fig. 4C). However, when the microsomal fraction of the mock-injected fat body of silkworm larvae was mixed into the purified hPPR−ΔTMΔCD with the ratio of 1:1, the binding affinity recovered to 64% of the microsomal fraction of hPRR−ΔTMΔCD (Fig. 4D).

The solubilized microsomal fraction of both hPRR−ΔTMΔCD and ΔBmgp64−hPRR−ΔTMΔCD expressed in the fat body showed specific affinity, but, when they were purified, they lost the binding affinity. When microsomal fraction of

mock−injected fat body of silkworm larvae was added in the purified hPRR−ΔTMΔCD, its binding affinity was recovered. It is probable that an artificial transmembrane domain stabilizes the hPRR−ΔTMΔCD and native conformation may be structurally recovered. Otherwise, the microsomal fraction mixture contains some unknown factor (for example, substrate or lipid) that attaches to detergent−hPRR−micelles and either forms. Artificial transmembrane domain and an unknown factor appear to be absent following purification, thereby losing the binding affinity.

Mendrola *et al*. (16) reported that epidermal growth factor (ErB) receptor was stabilized by transmembrane domain interaction. Chiang and Knowles (17) reported that transmembrane domain interactions affected the stability of the extracellular domain of the human triphosphate diphosphohydrolase (NTPDase). However, Chen et al. (18) reported that when human granulocyte−macrophage colony−stimulating factor was expressed in silkworm pupae, its activity was lost due to its purification process.

In conclusion, we demonstrated that the native transmembrane domain of hPRR plays an important role in the mechanism of binding with human renin. The extracellular domain in the microsomal fraction of the fat body was observed to be bound with human renin whilst no affinity was observed after purification. This indicates that either any conformation change of hPRR due to deletion of transmembrane domain or unidentified factor in the microsomal fraction of the fat body may interact with the extracellular domain of hPRR. This clarification is highly important in elucidating the interaction of hPRR with its ligand.

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REFERENCES

1. **Nguyen, G., Delarue, F., Burcklé, C., Bouzhir, L., Giller, T., Sraer, J.D.:** Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. J. Clin. Invest., **109**, 1417–1427 (2002).
2. **Suzuki, F., Hayakawa, M., Nakagawa, T., Uddin, M.N., Ebihara, A., Iwasawa, A., Ishida, Y., Nakamura, Y., and Kazuo, M.:** Human prorenin has "Gate and Handle" regions for its non-proteolytic activation. J. Biol. Chem., **278,** 22217-22222 (2003).
3. **Ichihara, A., Hayashi, M., Kaneshiro, Y., Suzuki, F., Nakagawa, T., Tada, Y., Koura, Y., Nishiyama, A., Okada, H., Uddin, MN., Nabi, AH., Ishida, Y., Inagami, T., Saruta, T.:** Inhibition of diabetic nephropathy by a decoy peptide corresponding to the “handle” region for nonproteolytic activation of prorenin. J. Clin. Invest., **114**, 1128–1135 (2004).
4. **Prescott, G., Silversides, D.W., Reudelhuber, T.L.:** Tissue activity of circulating prorenin. Am. J. Hypertension, **15**, 280–285 (2002).
5. **Burcklé, C.A., Danser, A.H.J., Müller, D.N., Garrelds, I.M., Gasc, J.M., Popova, E., Plehm, R., Peters, J., Bader, M., Nguyen, G.:** Elevated blood pressure and heart rate in human renin receptor transgenic rats. Hypertension, **47**,

552−556 (2006).

6. **Zhang, W., Ding, T., Zhang, J., Su, J., Yu, J., Li, J., Li, F., Wang, C., Liu, N., Liu, X., Ma, X., Yao, L.:** Fusion expression of DDR2 extracellular domain in insect cells and its purification and function characterization. J. Cell. Biochem., **102**, 41−51 (2007).

7. **De Crescenzo, G., Pham, P.L., Durocher, Y., O'Connor−McCourt, M.D.:** Transforming growth factor−beta (TGF−beta) binding to the extracellular domain of the type II TGF−beta receptor: receptor capture on a biosensor surface using a new coiled−coil capture system demonstrates that avidity contributes significantly to high affinity binding. J. Mol. Biol., **328**, 1173−1183 (2003).

8. **Harikumar, K.G., Pinon, D.I., Miller, L.J.:** Transmembrane segment IV contributes a functionally important interface for oligomerization of the Class II G protein−coupled secretin receptor. J. Biol. Chem., **282**, 30363−30372 (2007).

9. **Chiang, W.C., Knowles, A.F.:** Transmembrane domain interactions affect the stability of the extracellular domain of the human NTPDase 2, Arch. Biochem. Biophys., **472**, 89−99 (2008).

10. **Xue, R., Wang, S., Qi, H., Song, Y., Wang, C., Li, F.:** Structure analysis of the fourth transmembrane domain of Nramp1 in model membranes. Biochim. Biophys. Acta., **1778,** 1442-1452 (2008).

11. **Du, D., Kato, T., Nabi, A.H., Suzuki, F., Park, E.Y.:** Expression of functional (pro)renin receptor in silkworm larvae using BmMNPV bacmid. Biotechnol. Appl. Biochem., **49**, 195−202 (2008).

12. **Kato, T., Kageshima, A., Suzuki, F., Park, E.Y.:** Expression and purification of human (pro)renin receptor in insect cells using baculovirus expression system. Protein. Expr. Purif., **58**, 242−248 (2008).
13. **Motohashi, T., Shimojima, T., Fukagawa, T., Maenaka, K., Park, E.Y.:** Efficient large−scale protein production of larvae and pupae of silkworm by *Bombyx mori* nuclear polyhedrosis virus bacmid system. Biochem. Biophys. Res. Commun., **326**, 564–569 (2005).
14. **Li, J., Huang, J.D., Wu, B.Y., Chen, Q.:** Effects of propyl gallate on interaction between TNF−α and sTNFR−I using an affinity biosensor. Acta. Pharmacol. Sin., **26**, 1212−1216 (2005).
15. **Nabi, A.H., Kageshima, A., Uddin, M.N., Nakagawa, T., Park, E.Y., Suzuki, F.:** Binding properties of rat prorenin and renin to the recombinant rat renin/prorenin receptor prepared by a baculovirus expression system. Int. J. Mol. Med., **18**, 483−488 (2006).
16. **Mendrola, J.M., Berger, M.B., King, M.C., and Lemmon, M.A.**: The single transmembrane domains of ErbB receptors self-associate in cell membranes. J. Biol. Chem., **277,** 4704-4712 (2002).
17. **Chiang, W.C. and Knowles, A.F.:** Transmembrane domain interactions affects the stability of the extracellular domain of the human NTPDase 2. Arch. Biochem. Biophys., **472,** 89-99 (2008).
18. **Chen, J., Nie, Z.M., Lü, Z.B., Zhu, C.G., Xu, C.Z., Jin, Y.F., Wu, X.F., Zhang, Y.Z.:** Large−scale purification of human granulocyte−macrophage

colony−stimulating factor expressed in *Bombyx mori* pupae. Appl. Biochem. Biotechnol., **141**, 149-160 (2007).

Figure legends

FIG. 1. Expression and Western blotting analysis of hPRR, hPRR−ΔCD, and hPRR−ΔTMΔCD. (A) Schematic diagram of the hPRR, hPRR−ΔCD, and hPRR−ΔTMΔCD fused with GFP_{uv}. (B) Western blotting analysis of the hemolymph of BmMNPV/hPRR−ΔTMΔCD bacmid− (lane 1) and BmMNPV/hPRR bacmid− injected silkworm larvae (lane 2). M and lane 3 denote the molecular marker and mock −injected larvae, respectively. Closed and open arrows indicate hPRR (69 kDa) and hPRR−ΔTMΔCD (64 kDa) fusion protein, respectively. (C) GFP_{uv} fluorescence analysis of fusion hPRR (lane 1, straight arrow) and hPRR−ΔCD (lane 2, dotted arrow) expressed in the fat body.

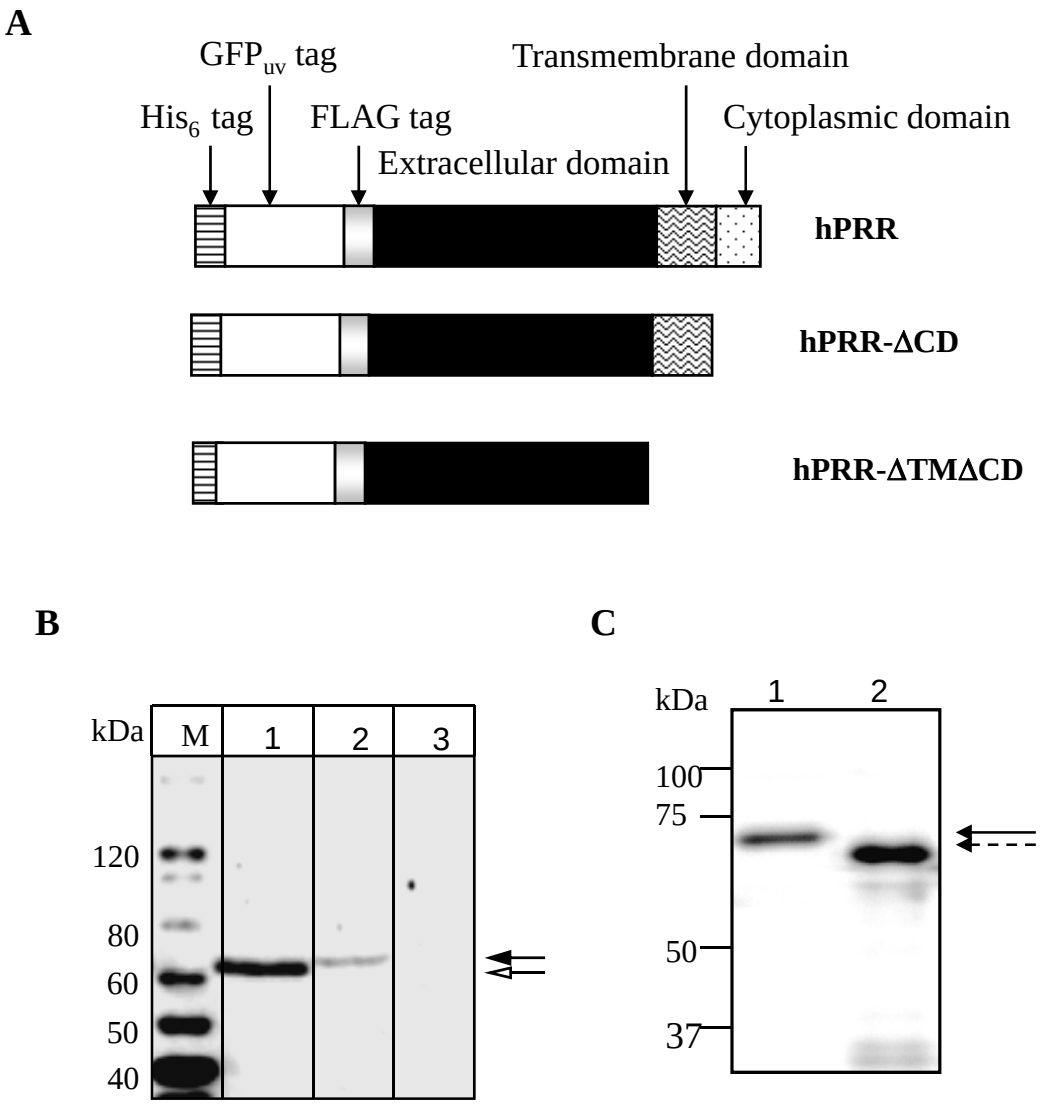
FIG. 2. Purification of three forms of hPRR, and binding assay for crude and purified proteins. (A) SDS−PAGE and GFP_{uv} fluorescence analysis of purified fusion hPRR, hPRR−ΔCD, and hPRR−ΔTMΔCD. Recombinant hPRR and hPRR−ΔCD were collected from the fat body, and hPRR−ΔTMΔCD from hemolymph, and they were purified. The resulting gels were either stained with Coomassie Brilliant blue (CBB) or visualized using a fluorescent imager by GFP_{uv}. Closed arrows with straight− and dotted−lines indicate purified hPRR and hPRR−ΔCD from the fat body, respectively. Open arrow indicates purified hPRR−ΔTMΔCD from hemolymph. (B) Three mg/ml of crude hPRR and hPRR−ΔTMΔCD were used for binding assay. (C) Purified hPRR and hPRR−ΔTMΔCD were diluted to 60 μg/ml by Buffer C, and used for its binding assay. Running buffer was injected as a control. (D) Evaluation of the dissociation equilibrium constant (K_D) of purified hPRR and hPRR−ΔCD bound to immobilized

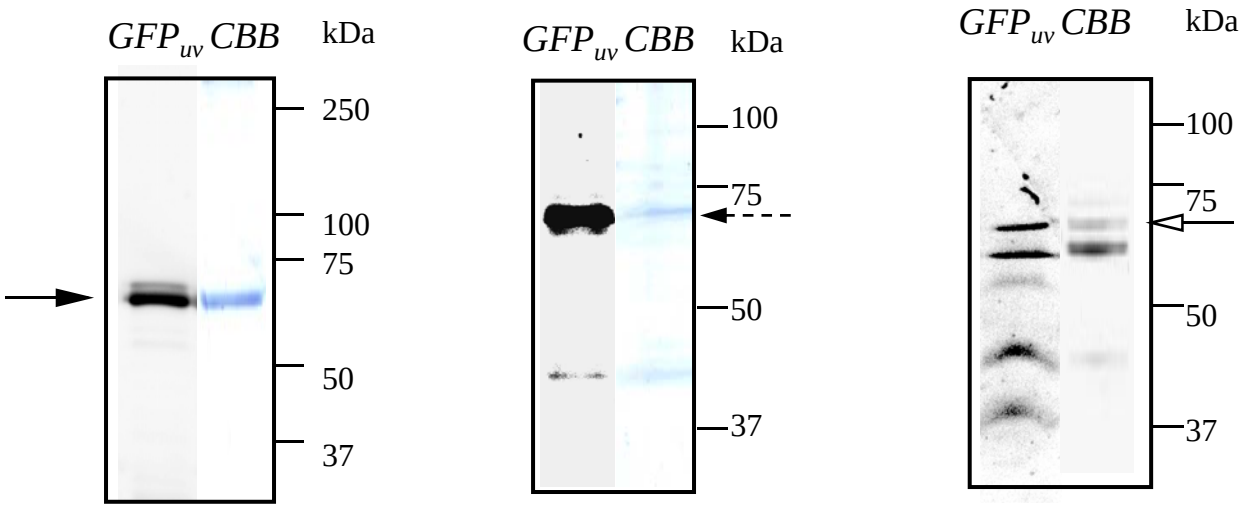
human renin by SPR. Sensorgram overlays of various concentrations of hPRR and hPRR−ΔCD injected over immobilized renin are shown. a, b, c and d denote the hPRR concentrations of 1, 0.5, 0.25 and 0.13 μM, respectively. e, f, g and h denote the hPRR −ΔCD concentrations of 1, 0.75, 0.5, and 0.25 μM, respectively. i indicates the running buffer as a control. K_D was estimated based on these results by the IAsys FASTfit software. The vertical arrows in B, C and D indicate the injection time.

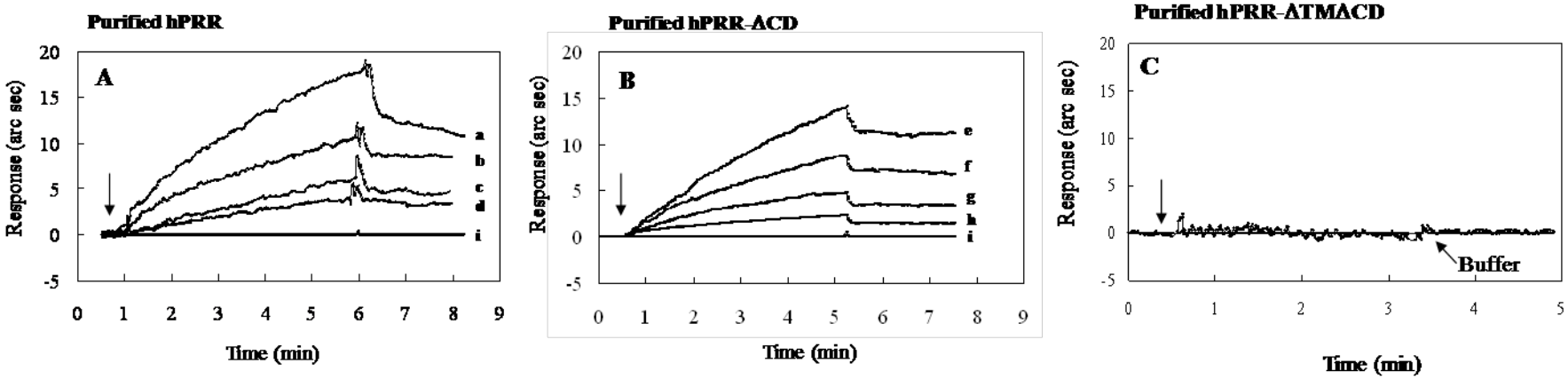
FIG. 3. Expression and binding assay of ΔBmgp64− hPRR−ΔTMΔCD. (A) Schematic diagram of ΔBmgp64−hPRR−ΔTMΔCD. (B) SDS−PAGE Western blotting of ΔBmgp64−hPRR−ΔTMΔCD expressed in the fat body of silkworm larvae. The arrow indicates the estimated molecular weight. (C) Binding assay of ΔBmgp64−hPRR−ΔTMΔCD in the microsomal fraction of the fat body and its corresponding fraction in the mock larvae. (D) Binding assay of purified ΔBmgp64−hPRR−ΔTMΔCD. The vertical arrows in C and D indicate the injection time. The concentration of protein was 60 μg/ml. Running buffer was injected as a control.

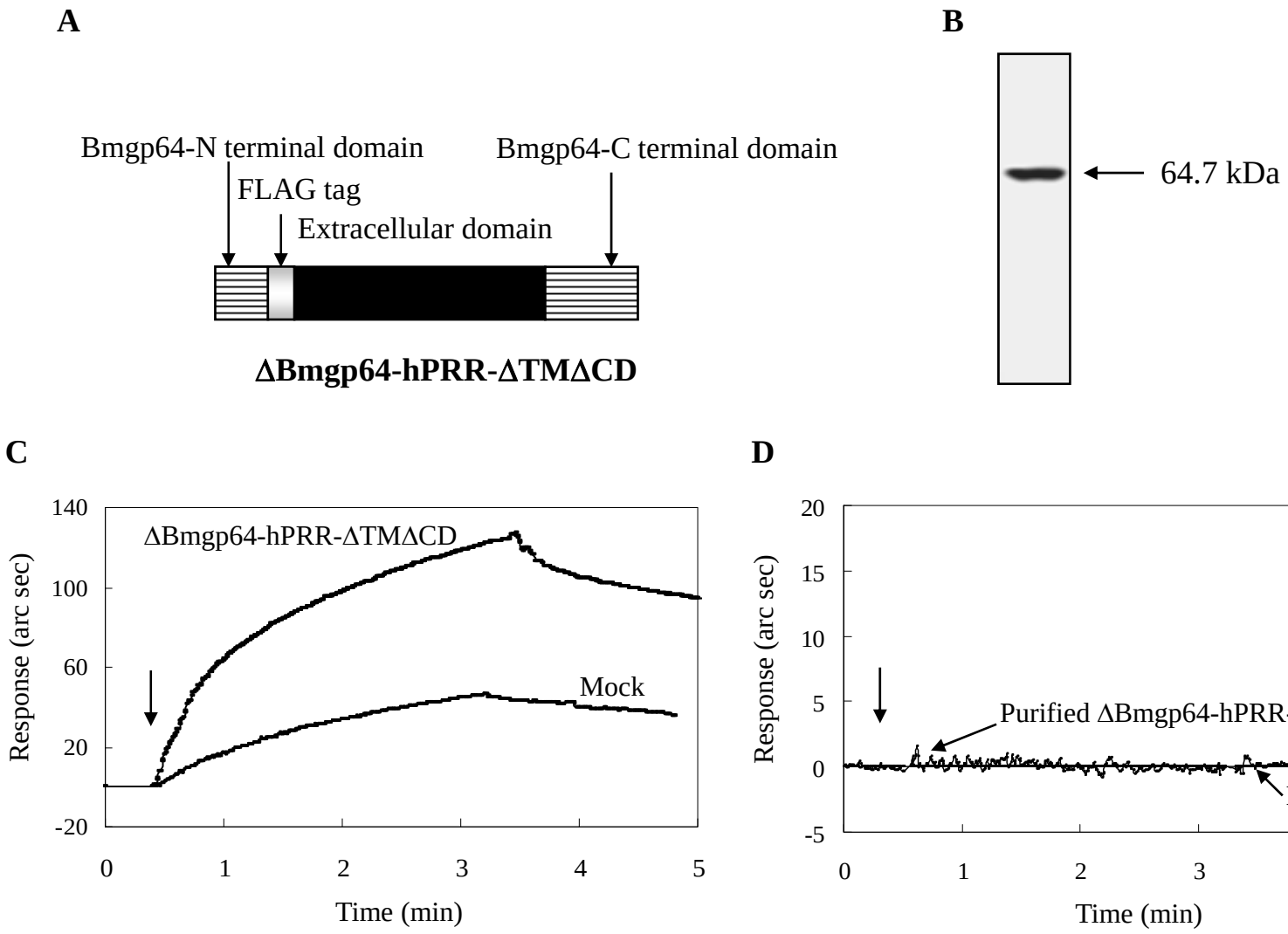
FIG. 4. Expression of hPRR−ΔTMΔCD in the fat body and its binding assay. (A) Western blotting of the microsomal and soluble fractions obtained from fat bodies expressing hPRR−ΔTMΔCD. (B) The microsomal fraction, soluble fraction, and their corresponding mock protein fractions were examined for their binding affinity at a concentration of 3 mg/ml. (C) Binding affinity of purified hPRR−ΔTMΔCD from the microsomal fraction of the fat body. The concentration of protein was 60 μg/ml. Running buffer was injected as a control. (D) Binding affinity of crude hPRR−ΔTMΔCD from the microsomal fraction of fat body (a), mixture of purified

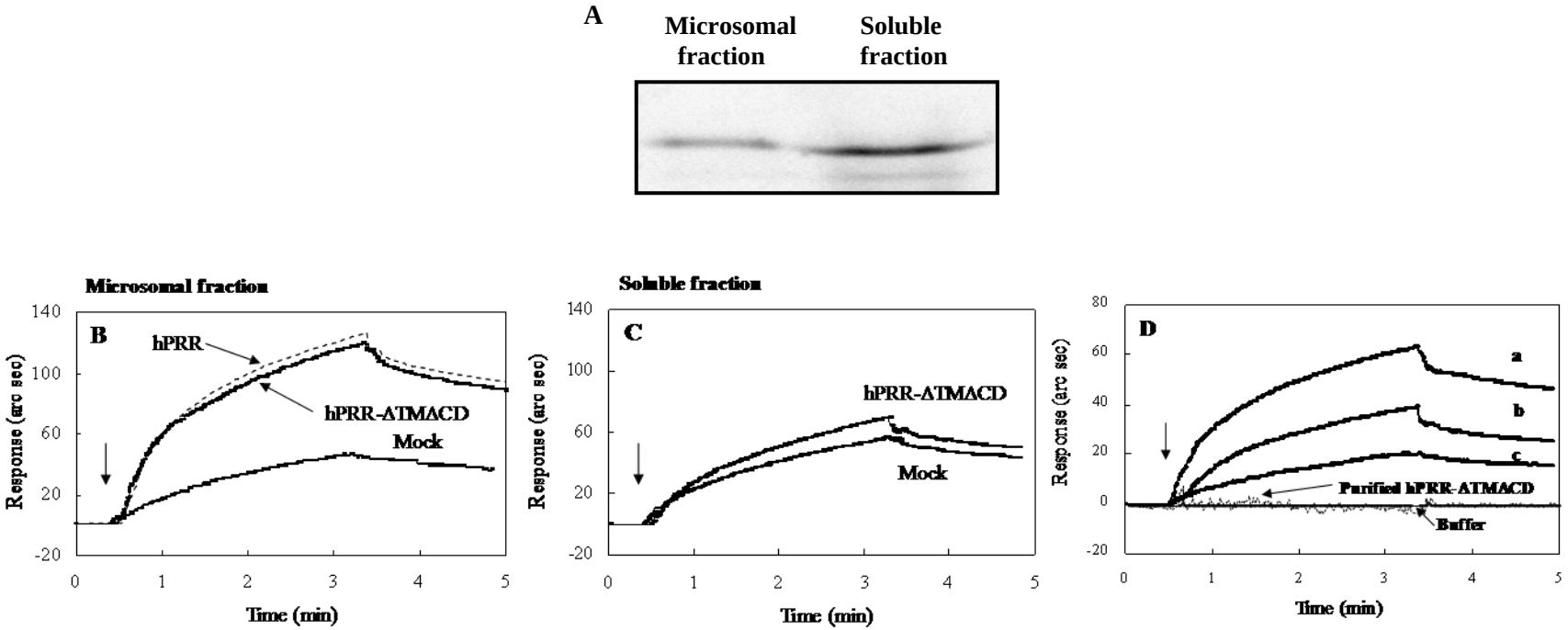
hPRR−ΔTMΔCD and microsomal fraction of fat body from mock−infected larvae with ratio of 1:1 (b), and its corresponding microsomal fraction of mock−infected fat body (c). The concentration of protein was 1.3 mg/ml. The vertical arrow indicates the injection time.


A
GFP$_{uv}$ tag
Transmembrane domain
His$_6$ tag
FLAG tag
Cytoplasmic domain
Extracellular domain
hPRR
hPRR-ΔCD
hPRR-ΔTMΔCD
B
kDa
M
1
2
3
120
80
60
50
40
C
kDa
1
2
100
75
50
37


GFP_uv CBB
kDa
250
100
75
50
37
hPRR
GFP_uv CBB
kDa
100
75
50
37
hPRR-ΔCD
GFP_uv CBB
kDa
100
75
50
37
hPRR-ΔTMΔCD


Purified hPRR
A
Response (arc sec)
Time (min)
a
b
c
d
i
Purified hPRR-ΔCD
B
Response (arc sec)
Time (min)
e
f
g
h
i
Purified hPRR-ΔTMΔCD
C
Response (arc sec)
Time (min)
Buffer


A
Bmgp64-N terminal domain
FLAG tag
Extracellular domain
Bmgp64-C terminal domain
ΔBmgp64-hPRR-ΔTMΔCD
B
64.7 kDa
C
ΔBmgp64-hPRR-ΔTMΔCD
Mock
Response (arc sec)
Time (min)
D
Purified ΔBmgp64-hPRR-ΔTMΔCD
Buffer
Response (arc sec)
Time (min)


A
Microsomal fraction
Soluble fraction
Microsomal fraction
B
hPRR
hPRR-ΔTMΔCD
Mock
Response (arc sec)
Time (min)
Soluble fraction
C
hPRR-ΔTMΔCD
Mock
Response (arc sec)
Time (min)
D
a
b
c
Purified hPRR-ΔTMΔCD
Buffer
Response (arc sec)
Time (min)

Supplementary file 1

For the solubility test, the following non-ionic detergents were tested: DDM, n-octyl β-D-glucoside, Triton X-100, CHAPS, sodium cholate and sucrose monolaurate. These detergents were of the highest grade and purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). 1.2 ml of the microsomal fraction of the fat body (to 5 mg/ml) was incubated with various detergents (0.1- 3%, w/v) for 1 h at 4 °C. Samples of each homogenate (0.1 ml) were transferred to separate tubes as controls, and the remaining homogenate was centrifuged for 60 min at 114,000 x g at 4 °C. The aliquots from the homogenates, supernatants and pellets were analyzed by SDS-PAGE and Western blotting. Band intensity was quantified by Quantity ONE software (Bio-Rad, Hercules, CA, USA).

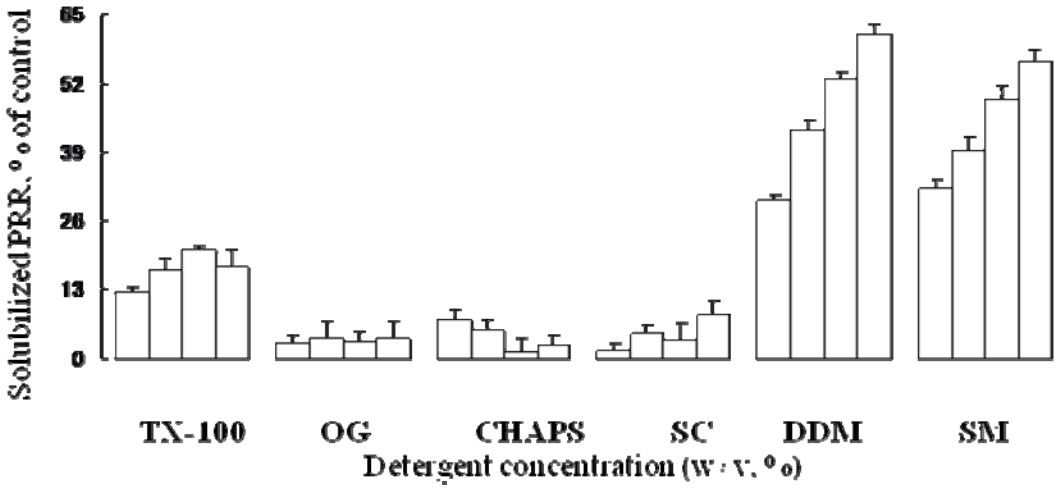


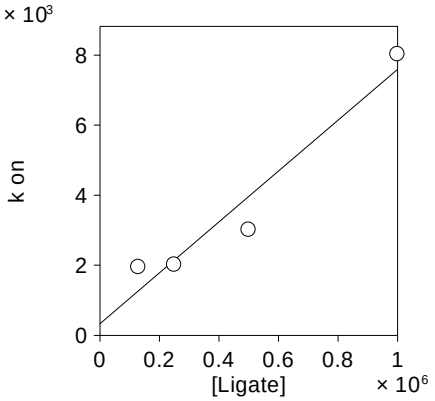
Fig. Result of detergent screening for solubilization of hPRR from the microsomal fraction

When DDM and sucrose monolaurate were used, more than 60% of the expressed protein was solubilized, as shown in the figure. DDM was used in this study for solubilization of hPRR from the microsomal fraction.

Supplementary file 2

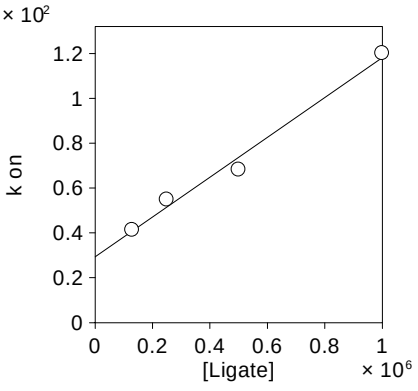
$$K_D = k_{diss}/k_{ass} = \text{intercept/gradient}$$

hPRR



Intercept = 0.000333712 ± 0.00076344
Gradient = 7259.15 ± 1324.27
Corr Coeff = 0.97

hPRR−ΔCD



Intercept = 0.00293281 ± 0.00041272
Gradient = 8882.78 ± 715.909
Corr Coeff = 0.99