Expression of protein complex comprising of the human prorenin and (pro)renin receptor in silkworm larvae using *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmids for improving biological function

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Abstract: Three forms of recombinant protein complexes comprising the human prorenin (hPro) and (pro)renin receptor (hPRR) (hPRR/prorenin) were successfully expressed in the silkworm larvae using *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmids. They were localized in the fat body cells and formed a prorenin-bound hPRR complex. The expressed levels of hPro and hPRR were similar judging from Western blotting. The hPRR/prorenin complex containing 40 μg of hPRR (yield, 43%) and 30 μg of hPro (yield, 34%) was purified from 15 silkworm larvae by a series of purification using anti-FLAG and Strep-Tactin affinity chromatography. The renin activity of the purified hPRR/prorenin complex was 3.8-fold that of the mixture of hPRR and hPro expressed individually *in vitro* judging from the renin assay. These results show that the unstable transmembrane protein, hPRR was coexpressed stably with ligand, hPro and formed a stable protein, hPRR/prorenin complex that showed a high catalytic active form.

Keywords Silkworm; *Bombyx mori* nucleopolyhedrovirus; Bacmid; (Pro)renin receptor; Prorenin/(pro)renin receptor complex

Introduction

Ligand–receptor complexes constituted by protein interaction appear to play a central role in most cellular functional pathways [1]. The challenge for structural biology research is how to coexpress the ligands and receptors efficiently in the same host cell and purify them in order to obtain more functional information at the molecular level. Baculovirus-infected insect cells and mammalian systems present an attractive expression system for providing and performing most of complicated posttranslational processing of the proteins, and sometimes required for the ligand–receptor complex formation. This strategy can be better than the standard *Escherichia coli* system, although it is usually seen as time-consuming, expensive and technically more challenging [2-4].

Nowadays silkworm larvae are used for expression system of eukaryotic proteins with complicate structure, because their protein expression level is 10- to 100-fold higher than that using insect cell. From these points of views, *Bombyx mori* nucleopolyhedrovirus (BmNPV) infected *B. mori* silkworm larvae or pupae are the most suitable combination for large-scale productions of eukaryotic proteins. Unfortunately, however, the traditional preparation of recombinant baculovirus that express exogenous genes needs at least 40 days, because multiple rounds of purification and amplification of viruses are needed. Recently, the bacmid (a baculovirus shuttle vector) system had been developed for BmNPV [5]. The BmNPV bacmid can be replicated in *E. coli* as a large plasmid and generate the recombinant virus DNA by the site specific transposition in *E. coli*, and remains infectious with insect cells and silkworm larvae. Since this

method eliminates multiple rounds of purification and amplification of virus, it greatly reduces both technical difficulty and time to select and purify recombinant viruses within 10 days. Therefore, the convenient bacmid system for BmNPV is using for as an important biotechnological method.

Renin–angiotensin system (RAS) plays an important role in the regulation of blood pressure, electrolyte balance and is also involved in renal, neuronal and endocrine functions related to cardiovascular control. Renin (EC 3.4.23.15) cleaves angiotensinogen to release the decapeptide angiotensin (ANG) I, which is further cleaved by ANG-converting enzyme to produce vasopressor peptide ANG II. Prorenin is the inactive precursor of renin, which has a prosegment with 43 amino acid residues attached at the N-terminus of renin. This prosegment has been considered to prevent interaction with angiotensinogen by covering the enzymatic cleft [6]. A (pro)renin receptor (PRR) bound prorenin with higher affinity than renin and displayed ANG I-generating activity without proteolytic removal of the prosegment [7]. Much physiological research has shown that the prorenin bound to PRR not only contributes to tissue ANG generation (particularly in subjects with diabetes), but also induces ANG-independent cardiovascular damage [8-10]. However, the interactions of prorenin with PRR and their structures have not been defined at the molecular level. Such efforts are critical to understand the molecular basis of ligand binding and for the ultimate goal towards the rational design of new receptor-blocking drugs. Insights into the three-dimensional structure of the receptor will facilitate the design of small ligands that can block PRR and be utilized therapeutically. Thus, the expression of the

hPRR/prorenin complex will be highly significant for determination of its crystal structure.

Recently, we expressed the full-length hPRR successfully in insect cells and silkworm larvae and showed its proper localization in the functioning form [11-12]. Here, we present a simple, cost-effective, rapid method for the expression and purification of the protein complex comprising of hPro and hPRR using silkworm larvae as a host. This approach provides a fast and affordable alternative strategy to produce mammalian proteins for structural and functional investigations. The hPro and hPRR were coexpressed stably in silkworm larvae and found to be formed as the protein hPRR/prorenin complex with a high biological active form.

Materials and methods

Construction of recombinant prorenin and its receptor bacmids

The DNAs encoding human prorenin were amplified from a human kidney cDNA library (Clontech, Palo Alto, CA, USA) by polymerase chain reaction (PCR) using KOD polymerase and primers (Table 1). All PCR reactions were performed using the following program: 3 min at 94°C, 35 cycles at 94°C for 15 s, 50°C for 30 s and 68°C for 90 s, followed by a final extension at 68°C for 5 min. The human prorenin (hPro) sequence was amplified using the forward primer (Primer 1) containing CACC and the native signal peptide sequences and the reverse primer (Primer 2) containing the related complementary sequence for encoding the C-terminal amino acids of hPro (Fig. 1). The

Strep II-tagged hPro (hPro-S) sequence was amplified using the forward (Primer 1) and reverse primers (Primer 3) encoding the complementary sequence of enterokinase (EK) recognition and Strep II tag sites upstream to Primer 2 (Fig. 1). In order to monitor the hPro expression and its localization *in vivo*, a far-red fluorescent protein (RFP)-fused hPro was constructed. The RFP sequence was amplified from pHcRed1 (Clontech) using Primers 4 and 5. Primers 4 and 5 were introduced at *Sph* I and *Sal* I restriction enzyme cleavage sites upstream to Primers 1 and 2, respectively. The resulting fragment was digested with *Sph* I/*Sal* I and ligated to pHcRed1, which was also digested with the same enzymes (phPro-RFP). The resulting phPro-RFP was amplified using Primers 1 and 6 to obtain hPro-RFP. The amplified hPro, hPro-S and hPro-RFP gene products were cloned into Gateway entry vectors using a pENTR/D-TOPO Cloning kit (Invitrogen, Carlsbad, CA, USA). After verification of the hPro DNA sequence, these entry clones were recombined with pDEST8 Gateway vector (Invitrogen) according to the manufacturer's protocol. The generated plasmids were transformed into *E. coli* BmDH10Bac-competent cells containing the BmNPV-CPD bacmid [13], from which the cysteine protease gene had been deleted. The resulting transformed *E. coli* BmDH10Bac cells were growing on LB agar (10 g of Bacto Tryptone, 10 g of Bacto Yeast Extract, 5 g of NaCl, and 15 g of agar per liter) plates containing 100 μg/ml of ampicillin, 50 μg/ml of kanamycin, 100 μg/ml of Blue-Gal, 7 μg/ml of gentamycin, 10 μg/ml of tetracycline, and 40 μg/ml of isopropyl-β-D-thiogalactopyranoside (IPTG). White antibiotics-resistant colonies were selected. Then, the BmNPV-CPD/hPro, BmNPV-CPD/hPro-S and BmNPV-CPD/hPro-RFP bacmids were isolated from each

112 transformed cells and their identities were confirmed by PCR using Primer 1 and each
113 corresponding reverse primer. The practical BmNPV bacmid system [5] is directly
114 applicable for the protein expression in silkworm. By using this system, target genes are
115 introduced into the BmNPV bacmids by site-specific transposition and are under the
116 control of polyhedrin promoter. The target protein can be successfully expressed in
117 silkworm larvae and pupae not only by direct injection of its bacmid DNA but also by
118 infection of its recombinant virus. Then The BmNPV-CPD/hPRR bacmid encoding
119 (pro)renin receptor (hPRR) was prepared as described [11], to which bombyxin signal
120 peptide, the UV-responsive green fluorescence protein (GFP_{uv}), enterokinase (EK) and
121 FLAG sites were fused in orderly sequence at the *N*-terminus of hPRR (Fig. 1).

122 Coexpression and localization of hPro and hPRR

123 Fifth-instar *B. mori* silkworm larvae (Ehime Sansyu, Co. Ltd., Yahatahama, Japan) were
124 reared in a 25°C incubator and each was injected with 50 μl of bacmid solution. The
125 bacmid solution consisted of 2.5 μg BmNPV-CPD/hPRR for (pro)renin receptor
126 expression and 2.5 μg one of the relevant bacmids (BmNPV-CPD/hPro,
127 BmNPV-CPD/hPro-S and BmNPV-CPD/hPro-RFP) for hPro expression, and 5 μl
128 transfection reagent (DMRIE−C, Invitrogen) in 45 μl of PBS (8 g NaCl, 0.2 g KCl, 1.4
129 g $Na_2HPO_4 \cdot 2H_2O$ and 0.24 g KH_2PO_4 in 1 L of distilled water, pH 6.8). Half an hour
130 after the injection, the larvae were fed with an artificial diet (Silkmate 2S, Nihon Nosan
131 Co. Ltd., Yokohama, Japan) and reared for 6 days further. To investigate the localization
132 of expressed proteins, the fat body was collected from silkworm larvae and immediately

observed using a confocal laser scanning microscope (TCS-LS, Leica Microsystem, Heidelberg, Germany) equipped with an imaging system.

Preparation of solubilized microsomal fraction from fat body cells

The fat body collected at 6 days postinjection was sonicated in homogenization buffer (pH 7.4; Buffer A) comprising 50 mM sodium phosphate, 150 mM NaCl and protease inhibitors (Roche, Lewes, Sussex, UK). Undisrupted cells and cellular debris were removed by centrifugation at 600 *g* for 10 min followed by a second centrifugation of the supernatant at 8,000 *g* for 30 min. The microsomal fraction was collected from the resulting pellet by centrifugation at 114,000 *g* for 60 min and was then washed twice with Buffer A. The pelleted microsomal 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 protein concentration of 5 mg/ml. Solubilized membrane proteins were collected from the supernatant after further centrifugation at 114,000 *g* for 60 min.

Coimmunoprecipitation analysis

Coimmunoprecipitation assays were carried out using the Catch and Release version 2.0 Reversible Immunoprecipitation System (Millipore, Billerica, MA, USA). Five hundred micrograms of solubilized microsomal fraction and either 4 μg of anti-hPro or anti-FLAG antibody were loaded onto the spin columns containing 0.5 ml of immunoprecipitation capture resin for 1 h at 4°C with gentle agitation. Columns were

153 washed three times, followed by sample elution. The eluates were subjected to sodium
154 dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) separation.
155 Fluorescence analysis of GFP_{uv} using a Molecular-FX multi-imager (Bio-Rad, Hercules,
156 CA, USA), or Western blotting analysis using an anti-hPro antibody [14] that recognizes
157 the hPro prosegment were performed. In additional negative control experiments,
158 microsomal fractions containing of single expressions of hPRR or hPro were used in the
159 immunoprecipitation steps and each was detected with the antibody of its interacting
160 partner.

161 Purification of recombinant complex protein

162 Around 5 mg/ml of solubilized microsomal fraction was incubated in batches for 1.5 h
163 at 4°C with 1/30 fraction volume of anti-FLAG M2 antibody agarose resin
164 (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 1000 *g* for 2 min and
165 removal of the supernatant, the resin was loaded into a gravity flow column and washed
166 with a 10-resin-volume buffer (50 mM sodium phosphate, 150 mM NaCl, protease
167 inhibitors and 0.2% DDM, pH 8.0; Buffer C). Proteins were eluted with a
168 5-resin-volume of Buffer C containing 100 μg/ml of FLAG peptide. The elution from
169 the anti-Flag column containing the hPRR/prorenin complex was incubated for 1 h at
170 4°C with 300 μl of Strep-Tactin Superflow Resin (IBA GmbH, Göttingen, Germany).
171 After loading the resin into gravity flow column and removing the flow-through, the
172 resin was washed with a 3-resin-volume of Buffer C. Proteins were eluted in a
173 3-resin-volume of Buffer C containing 2.5 mM desthiobiotin.

174 SDS–PAGE and Western blotting analysis

175 Silkworm larvae at 6 days postinjection (d.p.i.) were bled by cutting the abdominal legs
176 with scissors. Hemolymph was immediately mixed with 5 μl of 200 mM
177 1-phenyl-2-thiourea, centrifuged at 9000 rpm for 10 min at 4^{o}C, and resulting
178 supernatant was used for analysis. The larval fat body was isolated by dissection under
179 sterile conditions at 4^{o}C. It was then diluted with 25% (v/v) homogenization buffer
180 (0.15-M NaCl, 2-mM EDTA, 2-mM dithiothreitol (DTT) and 20-mM Na_2HPO_4; pH
181 7.6) and sonicated on ice three times for 15 s each, with 15-s intervals, using a sonicator
182 (VC 130PB, Sonic & Materials, Newtown, CT, USA). Finally, it was centrifuged at
183 13000 rpm for 30 min and resultant supernatant was used for analysis.

184 The fusion proteins contained in the hemolymph and fat body were detected by
185 SDS–PAGE and Western blotting. SDS–PAGE was performed with 12%
186 polyacrylamide gel using the Mini-PROTEAN II system (Bio-Rad). The respective
187 bands were detected using a Molecular-FX multi-imager (Bio-Rad). For Western
188 blotting, the samples were heated at 90°C for 10 min before running them on the
189 SDS–PAGE gel. Mouse anti-FLAG-M2 as the primary antibody (1:20000, Sigma) and
190 the anti-mouse IgG/HRP (horseradish peroxidase conjugated) antibody (1:20000, GE
191 Healthcare, Buckinghamshire, UK) as the secondary antibody were used for detecting
192 hPRR. Rabbit anti-hPro prosegment (1:5000) as primary antibody and the anti-rabbit
193 IgG/HRP (1:20000, GE Healthcare) as a secondary antibody were used for detecting
194 hPro. The immunoblot bands were visualized using enhanced chemoluminescence

(ECL) plus Western blotting detection reagents (GE Healthcare) and analyzed using a Fluor-S/MAX multi-imager (Bio-Rad).

Assay of renin activity and protein concentration

Renin activity was measured using a SensoLyte 520 Renin Assay Kit (AnaSpec, Inc., San Jose, CA, USA). The 5-FAM/QXL 520 fluorescence resonance energy transfer (FRET) peptide is cleaved by renin and releases the fluorescence of 5-FAM, of which signal is monitored for the renin activity. Briefly, the purified samples were diluted to 50 μg/ml and seeded into a 96-well plate at 100 μl/well and incubated at 37°C for 30 min. At the same time, a 50 μl aliquot of preincubated the renin substrate solution containing 5-FAM/QXL 520 FRET peptide at 37°C was added to each well. After mixing the reagents completely by shaking the plate gently for 25 s, the released fluorescence signal of 5-FAM was measured at excitation/emission of 490/520 nm using Fluoromark (Bio-Rad). The fluorescence readings were represented as relative fluorescence units (RFU). The protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) and bovine serum albumin as the standard.

Results

Coexpression of hPro and hPRR in silkworm larvae

When silkworm larvae were co-injected with BmNPV/hPRR and bacmids expressing

215 hPro, hPRR expression was confirmed by observing the intensity of GFP_{uv} under an
216 ultraviolet illuminator (Fig. 2A). The observed green fluorescence image indicated that
217 the GFP_{uv}-hPRR fusion product was expressed in the fat body. Three forms of hPro
218 were coexpressed with hPRR and analyzed by Western blotting analysis using an
219 anti-hPro antibody that recognizes the prosegment region. Three types of recombinant
220 hPro, hPro, hPro-S and hPro-RFP were detected, with estimated molecular weights of
221 42, 44 and 70 kDa, respectively (Fig. 2B). However, the recombinant hPro was not
222 detected in the hemolymph (Fig. 2C), indicating that hPro had accumulated specifically
223 in fat body cells. A specific band of commercial renin (45 kDa) was not detected
224 because it lacks the prosegment of hPro. This result revealed that the three forms of
225 recombinant hPro were each expressed intact in the fat body of silkworm larvae.
226 Combining together, these results indicate that hPRR and three forms of hPro were
227 successfully coexpressed in silkworm larvae.

228 Localization of human hPro and hPRR

229 Equal weights of two recombinant BmNPV/hPRR and BmNPV/hPro-RFP bacmids
230 were injected into the larvae and expressed proteins were observed using confocal laser
231 scanning microscopy. Some of the cells only expressed hPro and some only expressed
232 hPRR, but most expressed both hPro and hPRR (Fig. 3A–C). The hPRR was detected
233 with GFP_{uv} fluorescence (Fig. 3A), while hPro was also detected with red fluorescence
234 (Fig. 3B). Two images were merged in yellow color (Fig. 3C), suggesting that two
235 proteins were colocalized in fat body cells of silkworm larvae. In our previous report we

236 demonstrated that hPRR fusion protein was localized in the microsomal fraction of fat
237 body cells [11]. The expressed hPRR and hPro are assumed to be localized in the
238 membranes of the fat body cells.

239 To confirm the expression of protein complex of hPRR/prorenin, the solubilized
240 microsomal fraction was prepared and subjected to coimmunoprecipitation with either
241 anti-FLAG or anti-hPro antibodies. The eluates that was immunoprecipitated by
242 anti-FLAG were also analyzed by Western blotting with anti-hPro antibody (Fig. 3D).
243 Alternatively, the eluates that were immunoprecipitated by anti-hPro were analyzed by
244 SDS–PAGE and visualized using a Molecular-FX multi-imager for detecting GFP_{uv}
245 fluorescence (Fig. 3E). The hPRR and hPro proteins were each specifically
246 immunoprecipitated with the appropriate antibody, suggesting that the hPRR/prorenin
247 complex was formed in the fat body by coexpression of hPro and hPRR.

248 Purification of the hPRR/prorenin complex

249 The Strep II-tagged hPro and FLAG-tagged hPRR were coexpressed and purified by a
250 series of purification using anti-Flag M2 antibody and Strep-Tactin superflow
251 chromatography. This two-step affinity strategy can prevent contamination from hPro or
252 hPRR proteins expressed individually in the purified protein complex during each
253 affinity purification process. Solubilized microsomal fraction together with the nonionic
254 detergent DDM was incubated with an anti-FLAG bearing agarose. The hPRR and
255 hPRR/prorenin complex bound to the gel were eluted with FLAG peptide solution with
256 recovery yield of 85% of hPRR and 75% of hPRR/prorenin complex, respectively

(Table 2). The eluates were then incubated with Strep-Tactin resin and the hPRR/prorenin complex were eluted to yield the complex containing about 40 μg of hPRR and 30 μg of Strep II-tagged hPro, with recovery yields of 43% and 34%, respectively, This recovery of protein was obtained from 133 mg of solubilized microsomal protein extracted from 15 silkworm larvae (Table 2). The stoichiometric ratio of hPRR/prorenin was almost 1:1.

The elution fraction produced by anti-FLAG chromatography displayed a major band at 69 kDa by Coomassie Brilliant Blue (CBB) staining (Lane 1, Fig. 4A). After further fractionation by Strep-Tactin affinity chromatography, two bands at 69 kDa and 44 kDa were detected (Lane 2, Fig. 4A). Western blotting analysis with anti-hPro and anti-FLAG antibodies revealed that the hPRR/prorenin complex appeared quite stable (Lanes 1 and 2, Fig. 4B).

Functional analysis of the hPRR/prorenin complex

It has been reported that the hPro bound with hPRR becomes fully active by a conformational change of the molecule after the prosegment is opened [7, 15]. Therefore, renin activity was measured to confirm the conformational change of the purified hPRR/prorenin complex. The hPRR/prorenin complex showed a 3.8-fold level of specific renin activity compared with a mixture of hPro and hPRR (Fig. 5). Catalytic rate of hPRR/prorenin complex was 161.7 RFU/min, while that of mixture of individually expressed hPro and hPRR was 41.7 RFU/min. hPRR and hPro alone didn’t show catalytic activity of renin, 7 RFU/min. This indicates that, when hPro and hPRR

were coexpressed, the expressed hPro and hPRR in the fat body cells formed a hPRR/prorenin complex, which showed renin activity. Moreover, the protein complex was expressed stably as a biologically active form and was purified with a high recovery. This coexpression system with two different kinds of bacmid is effective for the maintenance of protein stability and purification by forming a ligand–receptor complex. Moreover, this hPRR/prorenin complex might be useful for further crystallographic studies.

Discussion

Structure of extracellular domain of receptor usually has a binding affinity with the receptor's ligand [16, 17]. Therefore, expression of the typically soluble extracellular domain of a receptor, rather than its full−length form, is employed to study the functions of a membrane protein. Through this approach, the use of detergents can be avoided and research on protein structure and function would be facilitated.

However, when the hPRR was expressed in silkworm larvae, it was located in the larval fat body, because of the hPRR composing of an extracellular domain, a transmembrane domain and a cytoplasmic domain [11]. We investigated the binding affinity of full−length of hPRR, hPRR lacking cytoplasmic domain, and the extracellular domain of hPRR. Interestingly, the transmembrane domain of hPRR is indispensable in the formation of functional hPRR [18]. 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. However, when microsomal fraction of mock−injected fat body of silkworm larvae was added in the purified extracellular domain of hPRR, its binding affinity was recovered. It is probable that the purification of hPRR causes conformational change of extracellular domain of hPRR and an artificial transmembrane domain stabilizes the extracellular domain of hPRR. Mendrola *et al*. [19] reported that epidermal growth factor (ErB) receptor was stabilized by transmembrane domain interaction. Chiang and Knowles [20] reported that transmembrane domain interactions affected the stability of the extracellular domain of the human triphosphate diphosphohydrolase (NTPDase).

To avoid this unstable expression of hPRR, here, we expressed successfully the hPRR/prorenin complex using silkworm larvae and purified it efficiently. BmNPV/hPro and BmNPV/hPRR bacmids were coinjected to silkworm larvae, which made it possible to express the hPRR/prorenin complex stably. The expressed hPRR/prorenin complex was purified using two different kinds of affinity chromatography without contaminating hPRR and hPro. This approach provides a fast and affordable alternative strategy to produce stably mammalian proteins for structural and functional investigations. This is the first report on the coexpression and purification of an active hPRR/prorenin complex using a silkworm larva-based expression system. These results will assist in studying the structural interactions of prorenin and PRR.

In conclusion, the protein complex was expressed stably as a biologically active form and was purified with a high recovery. This coexpression system with two different kinds of bacmid is effective for the maintenance of protein stability and

purification by forming a ligand–receptor complex. Moreover, this hPRR/prorenin complex might be useful for further crystallographic studies.

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Figure legends

Fig. 1 Genetic construction of human prorenin (hPro), Strep II-tagged human prorenin (hPro-S), HcRed-fused human prorenin (hPro-RFP) and GFP_{uv}-fused human (pro)renin receptor (GFP_{uv}-hPRR).

Fig. 2 Coexpression of recombinant hPRR and hPro in silkworm larvae. Coexpression of recombinant hPRR and hPro in silkworm larvae was confirmed by detecting GFP_{uv} fluorescence under an ultraviolet illuminator (A). Western blotting analysis of hPro from homogenate of fat body (B) and hemolymph (C) using anti-hPro prosegment antibody. Fifty μg and 15 μg of protein from homogenate of fat body and hemolymph were loaded, respectively. Lane 1, Molecular weight marker; lanes 2 and 3, commercial prorenin and renin, respectively; lane 4, hPro; lane 5, hPro-S; lane 6, hPro-RFP; lane 7, mock (injection with a BmNPV bacmid alone).

Fig. 3 Colocalization and coimmunoprecipitation of hPRR and hPro. Colocalized hPRR/pro-RFP in fat body cells were observed by confocal laser scanning microscopy. hPRR (A) and hPro-RFP were detected with GFP_{uv} fluorescence and HcRed fluorescence, respectively. Merging image of (A) and (B) is shown in (C). hPRR was immunoprecipitated with anti-FLAG antibody and followed by Western blotting with anti-hPro antibody (D). Prorenin was immunoprecipitated with anti-hPro antibody and followed by detection with GFP_{uv} fluorescence (E). Lane M, fluorescence molecular weight maker; lane 1, hPro/hPRR complex; lane 2, hPro-S/hPRR complex; lane 3,

418 hPro-RFP/hPRR complex. Closed and open arrows denote hPro and hPRR, respectively.
419 Scale bar indicates 20 μm.

420 **Fig. 4** Coomassie brilliant blue stained SDS–PAGE (A) and Western blotting (B)
421 analysis of the purified hPRR/prorenin complex by FLAG and Strep-Tactin affinity
422 resin. Lanes M, 1 and 2 denote molecular weight markers and eluates of FLAG affinity
423 and of Strep-Tactin affinity, respectively. Closed arrows, hPro; open arrows, PRR.

424 **Fig. 5** Catalytic activity of hPRR/prorenin complex, hPRR, hPro, and mixed sample
425 of hPRR and hPro. Fifty μg/ml of protein sample was used for measuring catalytic
426 activity using a SensoLyte 520 Renin Assay Kit. Symbols: closed circles,
427 hPRR/prorenin complex; closed triangles, mixed hPRR and hPro; closed squares,
428 hPRR; open triangles, hPro; open circles, reaction buffer.

Table 1 Gene-specific primers for PCR

Primer	Sequence
1	5′-CACCATGGATGGATGGAGAAGGATGC-3′
2	5′-TCAGCGGGCCAAGGCGAAGCCAATGCGGTTGTTACG CCGATCAAACTCTGTGTAG-3′
3	5′-TCACTTTTCGAACTGCGGGTGGCTCCACTTGTCGTCG TCATCGCGGGCCAAGGCGAAGCCAATGCGGTTG-3′
4	5′-TCAGCATGCACCATGGATGGATGGAGAAGGATG-3′
5	5′-TACGTCGACTTGTCGTCGTCATCGCGGGCCAAG-3′
6	5′-TCAGTTGGCCTTCTCGGGCAGGTCGCTG-3′

Table 2 Purification of the hPRR/prorenin complex

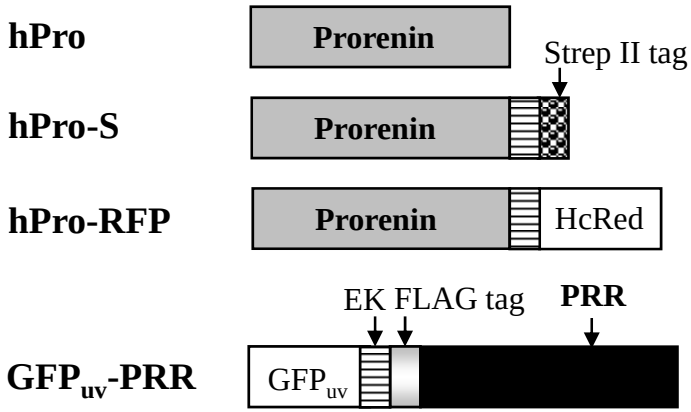
Purification stage	Total protein[a]	hPRR		hPro	
	(mg)	(μg)[b]	Yield (%)	(μg)[c]	Yield (%)
Solubilized microsome	133±11	94±08	100	89±10	100
Anti-Flag affinity		79±03	85	67±07	75
Strep-Tactin affinity		40±05	43	30±02	34

[a] Total protein as determined by BCA protein assay kit.

[b] hPRR determined by western blotting analysis using anti-Flag antibody with purified hPRR as a standard.

[c] hPro determined by Western blotting analysis using anti-hPro antibody with purified hPro as a standard. Various amounts (5, 10, 25, 25, 50, and 100 ng) of purified hPRR and standard hPro with sample loading were analyzed by Western blotting. Protein quantification was calculated by calibration curve correlated between intensity of immunoblot band visualized by ECL plus Western blotting detection reagent and its corresponding protein amount.

Data represent mean ± standard deviation from three independent experiments.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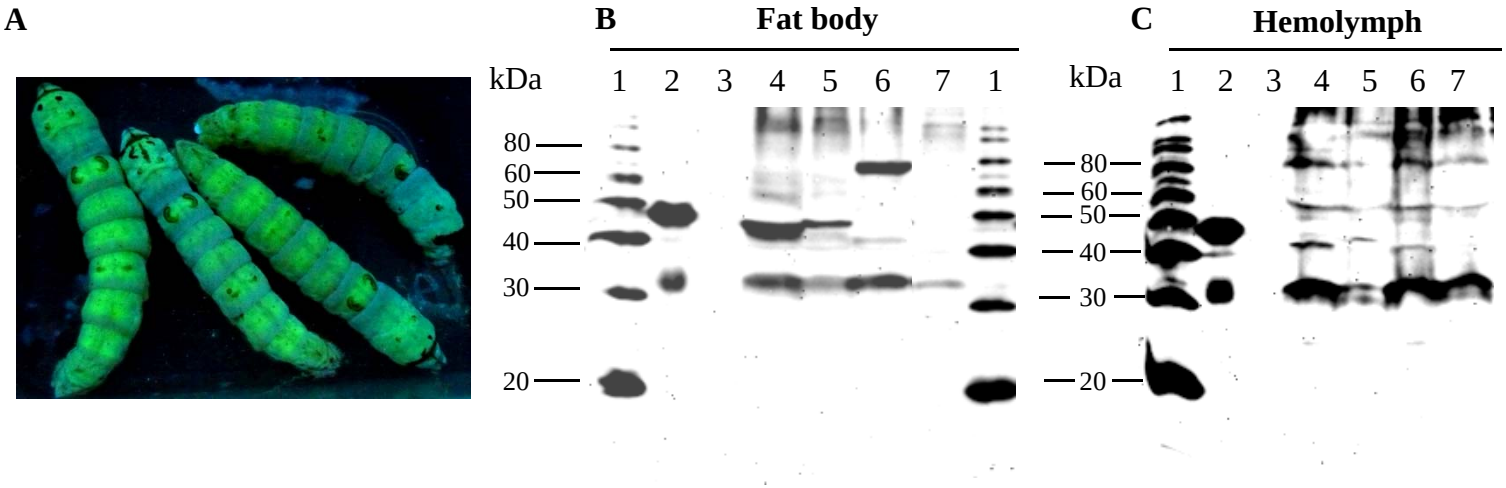
Strep II tag
hPro-S
Prorenin
hPro-RFP
Prorenin
HcRed
EK FLAG tag
PRR
GFPuv-PRR
GFPuv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B
Fat body
C
Hemolymph
kDa
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60
50
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30
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kDa
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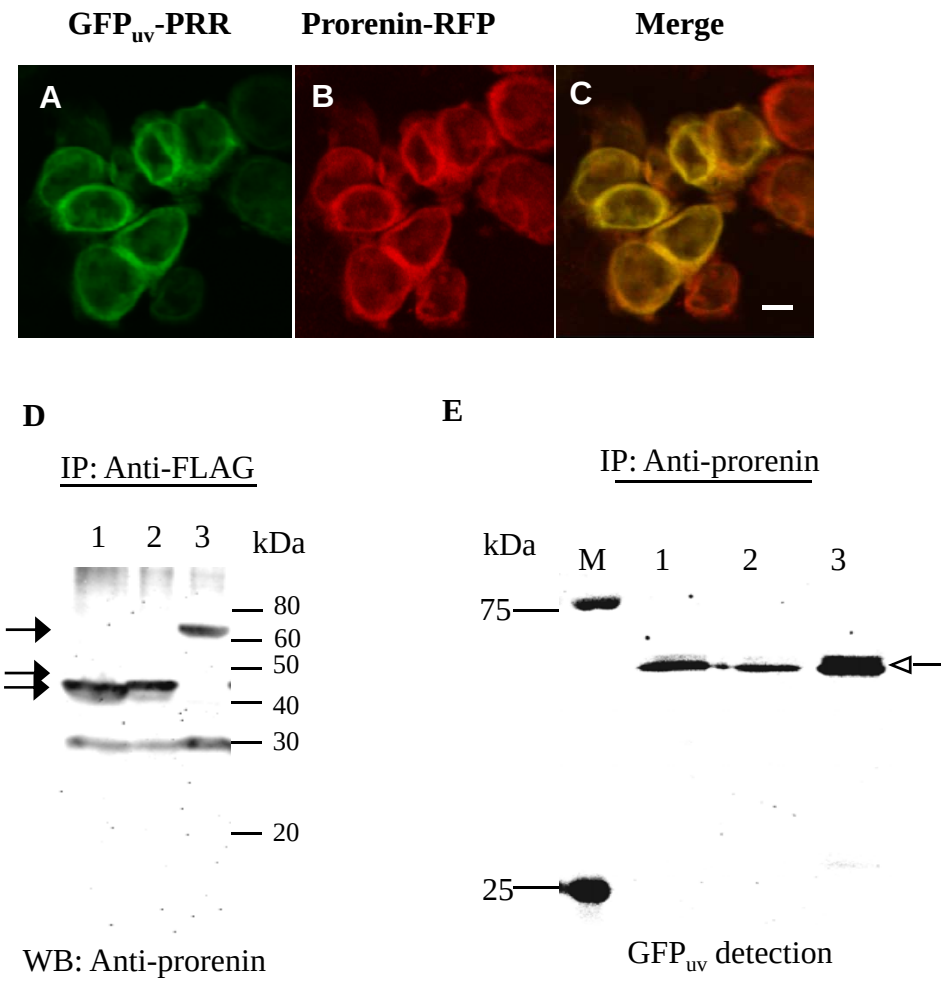
60
50
40
30
20


GFP$_{uv}$-PRR
Prorenin-RFP
Merge
A
B
C
D
IP: Anti-FLAG
1 2 3 kDa
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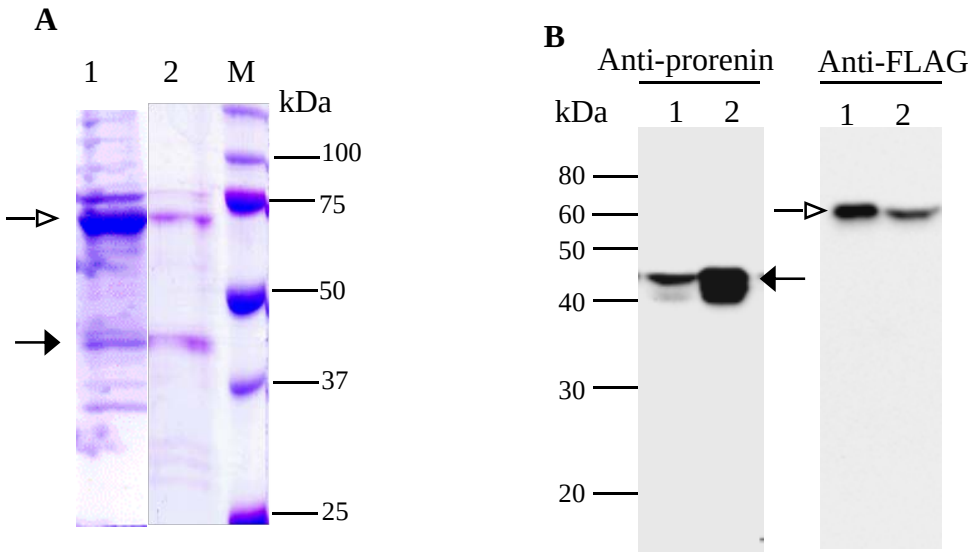
60
50
40
30
20
WB: Anti-prorenin
E
IP: Anti-prorenin
kDa M 1 2 3
75
25
GFP$_{uv}$ detection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2
M
kDa
100
75
50
37
25
B
Anti-prorenin
Anti-FLAG
kDa
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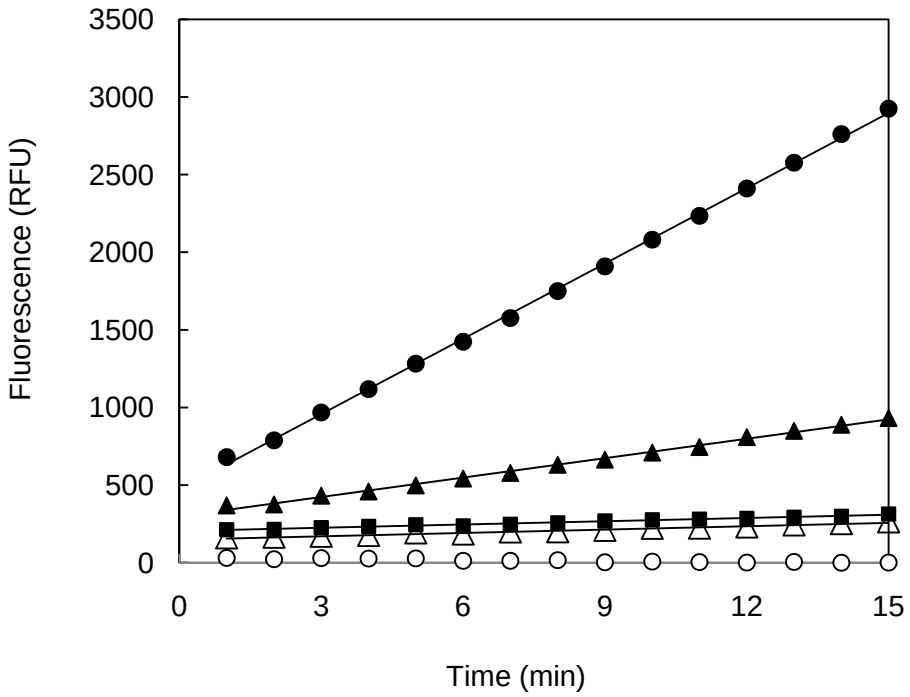
2
1
2
80
60
50
40
30
20


Fluorescence (RFU)
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2500
2000
1500
1000
500
0
0
3
6
9
12
15
Time (min)