Heliyon

Establishing a novel assay system for measuring renin concentration using cost effective recombinant ovine angiotensinogen

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Abstract

Background: Plasma renin can predict future cardiovascular events as well as the prevalence of chronic renal disease in hypertensive subjects. Ovine angiotensinogen (oANG) is a better substrate for measuring renin concentration through activity assay. Recombinant oANG expressed in *Escherichia coli* cells can be utilized as the substrate while measuring plasma renin. We aim to establish an immunoassay for measuring renin concentration at picomolar level using recombinant oANG.

Material and methods: Recombinant oANG was expressed in *E. coli* cells and purified to homogeneity. Various concentrations (0−1.5 pM) of recombinant human renin standard were prepared and incubated with recombinant oANG.

Renin activity was determined by angiotensin-I specific enzyme-linked immunosorbent assay.

Results: About 4.5 mg of purified recombinant oANG was obtained from 0.5 L of *E. coli* culture. The Michaelis constant and turnover number of human renin with recombinant oANG were 0.16 μM and 0.51 s^{-1}, respectively. A linear relationship was obtained when renin activity was plotted as a function of renin concentration using recombinant oANG as the renin substrate. Picomolar amounts of renin can be measured from known renin activity using this method.

Conclusion: This study established a novel assay system for measuring renin at picomolar level using cost effective recombinant oANG.

Keywords: Biochemistry, Biotechnology, Immunology

1. Introduction

Renin is the key enzyme of the renin–angiotensin–aldosterone system (RAAS) that maintains body fluid and thus, regulates blood pressure [1, 2]. This enzyme cleaves its substrate, angiotensinogen (ANG) to produce the decapeptide angiotensin I (Ang-I), followed by the enzymatic generation of octapeptide, angiotensin II that induces vasoconstriction, vasodilation and release of aldosterone [1, 2]. Dysfunction of RAAS activity may lead to organ damages leading to cardiovascular diseases [1, 2]. Plasma renin activity (PRA) measurement has gained recognition as a substitute of RAAS activity evaluation [3, 4, 5]. PRA has been suggested to be a predictor of the increased risk of cardiovascular diseases [6, 7, 8, 9, 10] and renal dysfunction [11, 12, 13].

A comparative study showed that among several laboratories, the reproducibility of plasma renin concentration (PRC) is higher than that of PRA [14]. PRC measured by activity assay (ac-PRC) is defined as Ang-I production by plasma renin enzymatic activity acting on saturating amounts of exogenous ANG [15]. Ovine ANG (oANG) has been utilized for many years as a renin substrate for measuring ac-PRC [16, 17]. Enzymatic studies showed that human renin has a higher affinity (lower K_m) for and higher reaction velocity (higher k_{cat}) with oANG than human ANG [18, 19, 20], where K_m and k_{cat} indicate Michaelis constant and turnover number, respectively. In other words, oANG has been shown to be a better substrate than human ANG in measuring ac-PRC [15, 21].

The initial rate of enzymatic reaction is proportional to the enzyme concentration under the saturating concentration of a substrate [22]. Thus, ac-PRC reflects the renin concentration. Owing to the lower K_m of oANG [18, 19, 20], a lower amount of ANG is needed to reach a saturating concentration of substrate when oANG is utilized in ac-PRC measurement. But the prerequisite of its measurement

is to prepare the enough amount of renin substrate, ANG. Native oANG used for ac-PRC measurement has been obtained from sheep plasma [16, 17]. Milligram amounts of oANG can be prepared as a recombinant protein using the Chinese hamster ovary (CHO) cell line [23]. Increasing the availability of oANG would facilitate the measurement of ac-PRC, which can be a measure of RAAS activity evaluation.

Recently, we have established an *E. coli*-based expression system for producing recombinant oANG [24], which showed 26-fold more time-effective production efficiency compared to our previously described CHO cell line-based production of ANG. Also, recombinant oANG produced by *E. coli*-based method retains a similar activity and stability to that expressed in CHO cells [24]. Since a large amount of oANG is available now, we take a step forward in the development of better ac-PRC assay system using our cost-effective recombinant oANG.

2. Materials and methods

2.1. Production of oANG using *E. coli* cells

The production of recombinant oANG in *E. coli* was performed as described previously [24]. Briefly, *E. coli* BL21 cells transformed with pTAC-oANG-His plasmid [24] were pre-cultured in LB medium supplemented with ampicillin. A preculture aliquot was inoculated into 0.5 L of Terrific Broth medium supplemented with a mixture of glucose and lactose, ampicillin, and antifoam. The cells were grown for 20 h at 30 °C using the culture system [24]. After the lysis of harvested cells, recombinant oANG was purified using immobilized metal affinity column chromatography followed by ion exchange column chromatography. The molar concentration of recombinant oANG was determined using the molecular extinction coefficient at 280 nm (39,165 M^{-1} cm^{-1}), which was calculated according to the formula provided by Kuramitsu *et al.* [25]. oANG (291 µM) dissolved in 20 mM Tris-HCl (pH 8.0) was diluted to 4 µM using 1× enzyme solution (ES) [10 mM NaH_2PO_4, 100 mM NaCl and 0.1% w/v BSA (fraction V); pH 7.2].

2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Fractions were pooled from different stages of recombinant oANG preparation and subjected to SDS-PAGE using SuperSep Ace 12.5% gel, 13 wells (Wako, Tokyo, Japan). Prior to SDS-PAGE, the protein samples were mixed with 5× loading dye and denatured at 96 °C for 5 min. Proteins were visualized via Coomassie Brilliant Blue (CBB) staining.

2.3. Differential scanning fluorimetry (DSF) analysis

DSF experiments were performed as described previously [26]. Briefly, purified recombinant oANG was diluted to 20 μM using phosphate buffer solution [8 mM Na_2HPO_4, 137 mM NaCl and 1.5 mM KH_2PO_4 (pH 7.5)]. Diluted recombinant oANG (20 μM) was mixed with SYPRO Orange solution [10×, 100 mM HEPES, 150 mM NaCl (pH 7.5)] in a 1:1 ratio. Fluorescence intensity of the mixture was measured using a StepOnePlus real-time PCR instrument (Applied Biosystems, Foster City, USA). To estimate the melting temperature (T_m), the Boltzmann equation was used to fit the fluorescence data using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA) and the calculation templates [26]. The T_m values were expressed as the mean ± standard deviation (SD).

2.4. Preparation of recombinant human renin standard

Recombinant human prorenin was expressed in CHO cells as described previously [20], and purified using ammonium sulfate precipitation followed by ion exchange column chromatography as previously described [24]. The protein concentration of human prorenin was determined using a molecular extinction coefficient at 280 nm of 47,705 M^{-1} cm^{-1}, which was calculated using the ProtParam tool [27]. The final concentration of human prorenin was 2.7 μM. Human prorenin was treated with trypsin to prepare human renin, as described previously [28]. The resulting human renin preparation was used as a recombinant human renin standard, and its aliquots were stored at -60 °C until use.

2.5. Enzymatic analysis

Various concentration (0.08−1.5 μM) of recombinant oANG were incubated with recombinant human renin standard (27 pM) at 37 °C for 30 min in 1× ES. The rate of Ang-I generation was determined by an enzyme-linked immunosorbent assay (ELISA) [29]. The K_m and maximum velocity (V_{max}) were estimated by Hanes-Woolf plot using GraphPad Prism 7.0. The k_{cat} of the renin-ANG reaction was calculated using the following formula:

$$k_{cat} = V_{max}/\text{renin concentration}$$

2.6. Establishment of calibration curve of renin concentration using recombinant oANG

Recombinant purified oANG (0.80 μM) and picomolar concentrations of human renin standard (0.20, 0.40, 0.60, 0.80, 1.0 and 1.5 pM) were reacted to measure the rate of Ang-I generation. The solution containing renin (5 μL), oANG (40 μL) and the 1× ES (155 μL) was incubated at 37 °C for 2 h. To the final volume

of 200 µL reaction mixture, 200 µL of ice cold 1× ES was added to stop the re-action between renin and oANG. Next, 100 µL of the diluted reaction mixture was added to each well and then an equal volume of Ang-I labelled with horseradish peroxidase (HRP) was added to the well. Thereafter, incubation for 2 h at 4 °C was followed by the addition of tetramethylbenzidine and H_2O_2 solution to develop color at 37 °C [29]. Finally, the HRP reaction was stopped by the addi-tion of 1 N H_2SO_4. Absorbance of the ELISA plate was recorded at 450 nm to measure the amount of Ang-I produced with respect to Ang-I standards (0.5−25 pg/µL) [29]. The Microplate Manager 6 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to calculate the amount of Ang-I produced from the absorbance. The enzymatic activity of renin (i.e., renin activity) is defined as follows:

Renin activity = [pg amount of Ang-I/(0.1 mL × 2 h ×1000)] ng Ang-I mL^{-1} h^{-1}

Calibration curve for measuring renin activity was plotted using mean $\pm$ SD values of six independent experiments (n = 6). Each experiment was performed in triplicates.

2.7. Statistical analyses

The data represented were expressed as mean $\pm$ SD where applicable. GraphPad Prism 7.0 was used to generate the figures where appropriate. Replicates of experi-ments required to construct each figure have been mentioned within the legends of respective figures.

3. Results and discussion

3.1. Production of recombinant oANG

Recombinant oANG was expressed in *E. coli* cells and purified to homogeneity us-ing two separate steps of column chromatography (Fig. 1A). The final preparation contained oANG protein alone (lane 5, Fig. 1A). About 300 µL of 291 µM recom-binant oANG was obtained from 0.5 L of *E. coli* cell culture, with a yield of about 9 mg per liter of culture. About four days were required from starting the cell culture to obtaining the final preparation.

To examine the quality of the protein, the absorbance of recombinant oANG (1:20 diluted) was measured with a scan range of 240−340 nm. The absorbance at 340 nm was almost zero (Fig. 1B), indicating that there was no aggregation in the protein preparation. The maximum absorbance was 0.562 at 279 nm, and the minimum absorbance was 0.015 at 340 nm.
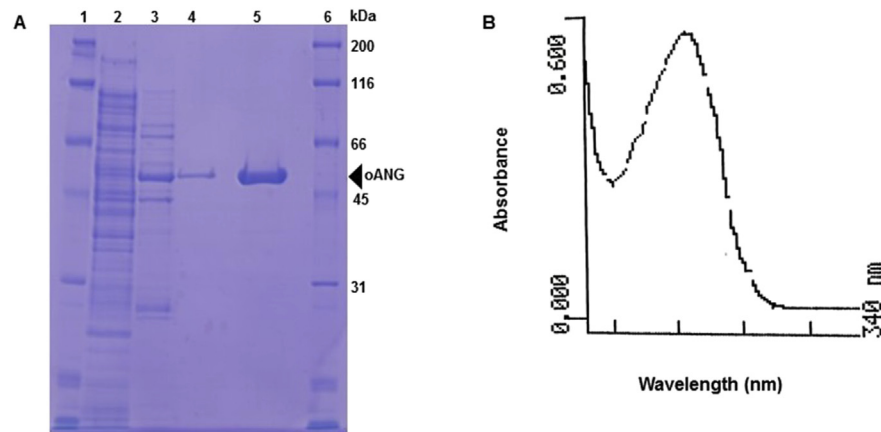
Fig. 1. (A) SDS-PAGE analysis of recombinant oANG. The stages of oANG production were separated by SDS-PAGE and stained with CBB. Lane 1, 6: molecular weight markers; Lane 2: cell supernatant after sonication; Lane 3: after affinity column chromatography; Lane 4: after ion-exchange column chromatography; Lane 5: concentrated oANG after purification (5 μg). (B) Spectroscopic analysis of oANG. The absorbance was measured with a scan range of 240−340 nm (10 mm path-length). Intervals at X-axis are 50 nm. The result was recorded with a thermal paper.

3.2. Protein stability analysis of recombinant oANG

DSF was used to examine the stability of recombinant oANG. This method monitors the thermal unfolding of proteins in the presence of a hydrophobic fluorescent dye and then the fluorescence intensity is plotted as a function of temperature [26]. The DSF results (Fig. 2) showed that oANG undergoes a two-state transition during the course of thermal denaturation. The T_m of recombinant oANG was 52.96 ± 0.22 °C.
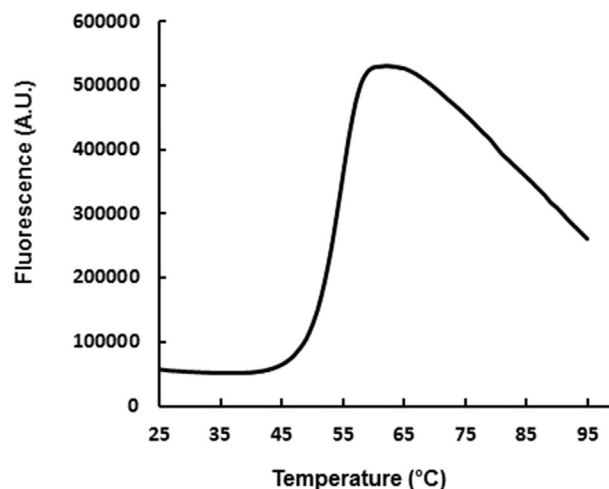
Fig. 2. Stability analysis of recombinant oANG. Recombinant oANG was analyzed by DSF. DSF data (n = 14) were measured with 10 μM oANG in 5× SYPRO Orange solution containing HEPES, Na_2HPO_4, KH_2PO_4 and NaCl (pH 7.5). One representative DSF plot is shown here, which was generated using GraphPad Prism software.

### 3.3. Preparation of recombinant human renin standard

Recombinant human prorenin was expressed in CHO cells and purified to homogeneity (Fig. 3). Prorenin is the inactive precursor of renin and contains a prosegment that masks the active site of renin [2, 15]. Proteolytic cleavage of the prosegment converts prorenin into renin [15], and thus, the conversion ratio of prorenin into renin is 1:1. Time course analysis of trypsin treatment showed that Ang-I-generating activity reached a plateau 7 min after the addition of trypsin (Fig. 4), indicating that all the prorenin was converted into renin. The resulting renin preparation (2.7 μM) was used as a recombinant human renin standard and further diluted for measuring renin activity.

3.4. Enzymatic analysis of recombinant oANG

A steady-state kinetic analysis was performed using recombinant oANG. As shown previously [24], recombinant oANG expressed in *E. coli* cells showed a typical substrate saturation curve (Fig. 5A). A plateau was obtained with 1.5 μM of recombinant oANG (Fig. 5A). A K_m value of 0.16 μM, V_{max} value of 0.76 nM min^{-1}, and k_{cat} value of 0.51 s^{-1} were obtained from the Hanes-Woolf plot (Fig. 5B).

3.5. Establishing the calibration curve of renin concentration using recombinant oANG

There are two approaches to define PRC [15]. One is PRC measured by immunoassay (ir-PRC), which is expressed as mass concentration. The other is ac-PRC, which is determined by measuring the enzymatic activity of renin with an excess amount of renin substrate to avoid the influence of variation in plasma ANG
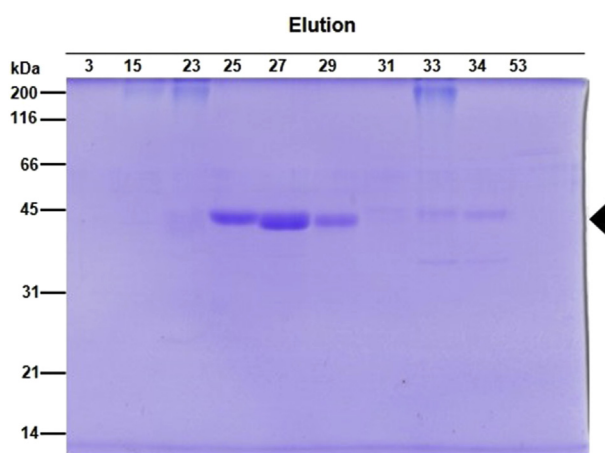
Fig. 3. SDS-PAGE analysis of recombinant human prorenin. The fractions obtained after Resource S column chromatography were subjected to SDS-PAGE followed by CBB staining. The migration distances of the marker proteins as well as their molecular weights are shown on the *left*. The arrowhead shown on the *right* indicates the size of the recombinant human prorenin.
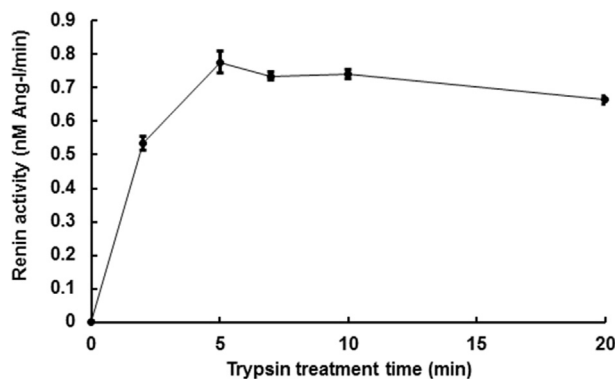
Fig. 4. Conversion of prorenin into renin by trypsin treatment (n = 3). Stable renin activity was obtained after 7 min of trypsin treatment. Renin activity decreased after 15 min. Error bars show the SD values ranging from 0.007-0.06.
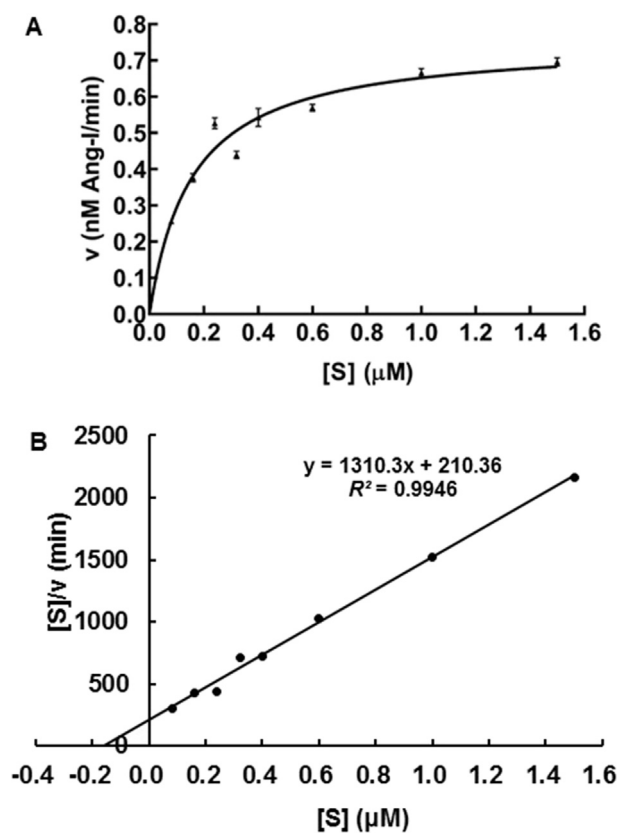
Fig. 5. Determination of K_m and $V_{max.}$ (A) [S]-V plot. Saturation of the reaction rate was indicated by the plateau at 1.5 μM of substrate concentration. Error bars show the SD values ranging from 0.005-0.025. GraphPad Prism software was used to plot the graph. (B) Hanes-Woolf plot.

concentration [15]. In this study, we aimed to establish a novel assay system for ac-PRC using our recombinant oANG.

Various concentrations of renin in the pM range were prepared using recombinant human renin standard. Renin activities were measured at 0.8 μM of oANG (5 times

Table 1. Determination of renin activity (ng Ang-I/mL/h) with varying renin concentration.

Renin concentration (pM)	Renin activity (ng Ang-I/mL/h)						Mean	SD	SEM	CV (%)
	Experiments (n = 6)									
	1	2	3	4	5	6				
0	0	0	0	0	0	0	0	0	0	0
0.20	0.414	0.433	0.417	0.271	0.321	0.411	0.38	0.07	0.03	17.44
0.40	0.897	0.847	0.863	0.908	0.956	0.884	0.89	0.04	0.02	4.28
0.60	1.271	1.239	1.336	1.201	1.310	1.323	1.28	0.05	0.02	4.13
0.80	1.685	1.533	1.595	1.848	1.997	1.888	1.76	0.18	0.07	10.31
1.0	2.317	2.239	2.285	2.010	2.203	2.317	2.23	0.12	0.05	5.21
1.5	3.179	3.079	3.098	3.059	2.933	3.118	3.08	0.08	0.03	2.66

SD, standard deviation; SEM, standard error of mean; CV, coefficient of variation.

higher than K_m value), which is comparable with saturating amounts of exogenous ANG. In each condition, less than 1% of oANG was consumed, which indicates that the initial velocities of the renin-ANG reaction were estimated properly. Using the data (Table 1), a linear calibration curve was generated by plotting the renin activity *versus* renin concentration (0.2−1.5 pM), and the value of determination coefficient (R^2) was calculated to be 0.99 (Fig. 6). This result suggests a strong linear relationship between renin activity and renin concentration. An intra-assay coefficient of variation (CV) of <20% was found in the six repeated experiments (Table 1), which
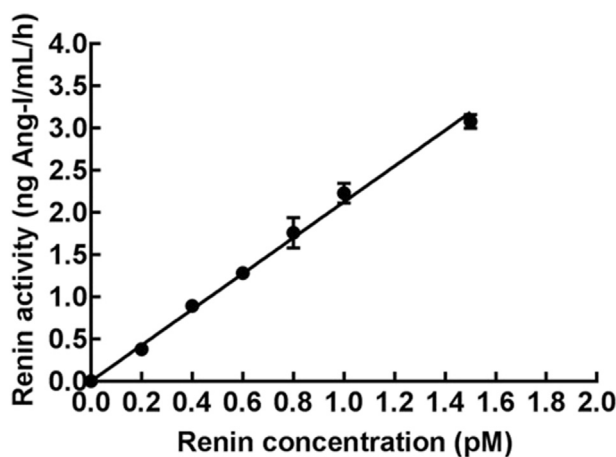
Fig. 6. Linear calibration curve plotting renin activity *versus* renin concentration. Calibration curve for measuring renin activity was plotted using mean ± SD values of six independent experiments (n = 6). Each experiment was performed in triplicates. Linear regression analysis produced a slope of 2.10, a y-intercept of 0.02, and a R^2 of 0.99 (n = 6) suggesting a linear relationship between renin concentration and renin activity. Error bars show the SD values ranging from 0.04-0.18. GraphPad Prism software was used to generate the graph.

is comparable with the reported data of functional sensitivity for the ir-PRC assay [30, 31].

Unknown renin concentration can be determined from known renin activity for any point on a linear calibration curve plotting renin activity *versus* renin concentration. Fig. 6 shows that our method can estimate renin concentrations ranging from 0.2 pM and above by selecting an appropriate dilution. Given that the molecular weight calculated from the amino acid sequence of renin is 37 k [27], the measurable range is 7.4 ng/L or above. The mean value of ir-PRC in healthy individuals is 13.8 ng/L, whereas that in healthy individuals administrated with aliskiren is 629 ng/L [15]. Unger *et al.* [32] reported that ir-PRC values (mean $\pm$ SD) in cases of essential hypertensives and healthy volunteers were 18.5 $\pm$ 36.5 ng/L and 12.7 $\pm$ 10.4 ng/L, respectively. Our method with recombinant oANG as a human renin substrate is applicable for measuring active renin concentration in human plasma.

3.6. Comparative studies on enzymatic properties of oANG

Skinner [16] prepared native oANG from nephrectomized sheep plasma, and reported that the rate of reaction of human renin with oANG is approximately five-times as fast as with human ANG at the same substrate concentration. This research also showed that the linear relationship between PRC and reaction rate is observed with oANG as the renin substrate, and proposed that renin assay systems with the addition of oANG is useful for routine determination of ac-PRC [16]. Since the Skinner's report, many researchers have used oANG for measuring ac-PRC [17, 20, 30, 33, 34, 35, 36].

The rationale for using oANG as human renin substrate is its higher rate of reaction with human renin. As a result of enzymatic investigations using native oANG and native human ANG, the K_m value of human renin-oANG reaction was reported to be 0.3 μM [18, 19] and 0.2 μM [21] whereas that of human renin-human ANG reaction was 1 μM [18, 19] and 2 μM [21]. When an excess amount of oANG is present, endogenous human ANG does not significantly affect the measured reaction rate of plasma renin [21, 37]. These enzymatic properties of oANG account for its advantage of measuring ac-PRC.

As part of understanding how oANG reacts with human renin, Nagase *et al.* [38] cloned cDNA of oANG and deduced the amino acid sequence. Milligram amounts of recombinant oANG were prepared using a CHO cell line [23]. Assayed with human renin, the K_m and k_{cat} values of recombinant oANG were reported to be 0.21 μM and 2.5 s^{-1}, respectively [20]. oANG shows a 4.4$\times$ lower K_m and a 2.1$\times$ higher k_{cat} than human ANG, thereby resulting in a 9$\times$ higher catalytic efficiency. Recombinant oANG expressed in CHO cells exhibits the differences in isoelectric points, and the differences are due to sialic acids attached to recombinant oANG [39]. Among the two potential N-linked glycosylation sites [38], amino acid residue at

position 271 is the most potential site in oANG [40]. Desialyated and deglycosylated oANG retain similar properties while reacting with renin as it was observed using intact recombinant oANG [39, 40]. These studies [38, 39, 40] indicate that fundamental properties of oANG are conferred by a polypeptide chain, and suggest the possibility that a polypeptide chain of oANG can be folded properly even in prokaryotic cells, where an oligosaccharide is not attached on proteins synthesized.

Recently, we established *E. coli*-based production of recombinant oANG, which allows us to prepare milligram amounts of the protein and is more time-effective than CHO cells-based one [24]. We carried out enzymatic analysis with recombinant human renin (25 pM) to show that oANG produced in *E. coli* cells is functionally comparable to oANG produced in CHO cells [24]. With the enhanced availability of recombinant oANG, achieved by the previous work [24], we carried out current study and established a linear relationship between renin activity and renin concentration (Fig. 6). The relationship is valid in the range of renin concentration from subpicomolar to picomolar level (0.2−1.5 pM), which covers the concentration levels observed in human plasma. In this study, we succeeded in enzymatic analysis for renin at a lower concentration than the previous study [24], and propose a new assay system amenable for estimating ac-PRC with recombinant oANG produced in *E. coli* cells. Fig. 7 demonstrates comparison of the properties of renin-ANG reaction between previous studies and current study along with their sources.

Native oANG prepared from sheep plasma have been utilized for measuring ac-PRC using human plasma [16, 17] and mouse plasma [41]. The previous study showed

Comparison of properties of renin-ANG reaction obtained from different sources

ANG	Source (ANG)	Renin	Source (Renin)	K_m (μM)	k_{cat} (s^{-1})	Reference
Sheep	Plasma	Human	Kidney	0.31	NM	[19]
Human	Plasma			1.15	NM	
Human	Plasma	Human	Recombinant (CHO)	0.91	1.2	[20]
Sheep	Recombinant (CHO)			0.21	2.5	
Sheep	Recombinant (*E. coli*)	Human	Recombinant (CHO)	0.07	0.59	[24]
Sheep	Recombinant (*E. coli*)	Human	Recombinant (CHO)	0.16	0.51	Current study

NM, not mentioned
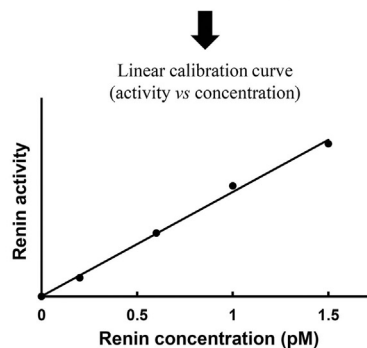
Fig. 7. Establishing a novel assay system for measuring renin concentration using cost effective recombinant oANG. Yamashita *et al.* [24] characterized renin at the level of 25 pM while the current study carried out enzymatic analysis of renin at sub-picomolar to picomolar (0.2−1.5 pM) ranges.

that oANG can work as a substrate for rat renin [23]. These findings indicate that oANG can be used as a universal renin substrate. The new assay system established in this study can be applicable not only for human plasma but also for mouse and rat plasma.

4. Conclusions

Renin concentration in plasma has significant prognostic value in patients with cardiovascular and renal diseases. This study proposes a novel assay system for measuring renin concentration using cost-effective recombinant oANG, which is produced in *E. coli* cells. Picomolar amounts of renin can be measured using this proposed assay, which can then be utilized in clinical assays and research purposes.

Declarations

Author contribution statement

Jobaida Akther: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A. H. M. Nurun Nabi, Tsutomu Nakagawa, Fumiaki Suzuki: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Satoshi Ohno, Takashi Yokogawa: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Akio Ebihara: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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