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# Functional characterization of Asp-317 mutant of human renin expressed in COS cells

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Renin is an unique aspartyl (acid) protease with optimal activity at neutral pH. It has been suggested that Ala-317 of human renin contributes to neutral optimum pH of the enzyme [(1984) FEBS Lett. 174, 102–111]. The hypothesis was verified by the characterization of mutant renin in which Ala-317 was replaced with Asp by a site-directed mutagenesis. Wild-type and mutant renins, which were expressed in COS cells, exhibited different pH-activity profiles and optimum pH of the mutant enzyme was lower than that of the wild-type enzyme. This result suggests that Ala-317 of human renin plays an important role in the determination of optimum pH of the enzyme.

Renin; Protein engineering; Optimum pH; Mutant expression; (COS cell, Human)

## 1. INTRODUCTION

Renin is an aspartyl protease with two catalytically important aspartic acid residues. This enzyme has unique characteristics in substrate specificity, optimum pH and physiological function among aspartyl proteases [2]. First, renin acts on only angiotensinogen to generate angiotensin I (AI), whereas other aspartyl proteases digest many protein substrates. Second, optimum pH of renin is neutral (pH 5.5–7.5), but those of other aspartyl proteases are acidic (pH 2.0–4.0).

From the alignment of amino acid sequences of renin and other aspartyl proteases, it has been suggested that Ala-317 of the human renin contributes a high optimum pH to the enzyme [1]. The alanine residue is commonly found in human [3], mouse [4] and rat [5] renin sequences, whereas in other aspartyl proteases the corresponding residue is substituted with aspartic acid (fig.1). As Ala-317 of the human renin is close to the catalytically important aspartic acid residues [1], it may affect the $pK_a$ of the aspartic acid residues and contribute to the unique optimum pH. The hypothesis was verified by the characterization of Asp-317 mutant renin expressed in COS cells.

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*Abbreviations:* COS-CM, COS cells conditioned medium; AI, angiotensin I; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; CHO cells, Chinese hamster ovary cells

## 2. MATERIALS AND METHODS

Human renin cDNA [3] was subcloned into pUC119 [13] and single-stranded DNA was prepared as described [13]. Site-directed mutagenesis was performed by the method of Sims et al. [14] using the single-stranded DNA as a template. DNA sequence of the mutated cDNA was determined to verify that only the desired mutation occurred. Wild-type human renin cDNA in pSVDPRnPA33 [15] was replaced with the mutated cDNA to generate plasmid pAD317SV in which the codon for Ala-317 was replaced with an Asp codon. pSV$\Delta$Rn was constructed by blunt-end ligation of a 5.2 kb *AvaI/Eco*RV fragment from pSVDPRnPA33. COS-7 cells were transfected with the plasmid by the DEAE-dextran method with chloroquine treatment [16]. Transfected cells were cultured in serum-free Dulbecco's modified Eagle's medium.

Partially purified human renal renin was obtained by the method of Haas et al. [17]. Hog angiotensinogen was purified as described [18]. Renin activity was determined by radioimmunoassay of AI generated during the enzymatic reaction [18]. Renin concentration was measured by using a Renin RIA

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Pasteur kit purchased from Diagnostics Pasteur. Anti-renin antiserum was obtained from a rabbit immunized with recombinant human renin from CHO cells [15], which was kindly supplied by The Upjohn Company. SDS-PAGE was performed by the method of Laemmli [19] and Western blot analysis was carried out as described [20].

## 3. RESULTS AND DISCUSSION

COS-7 cells were transfected with the respective expression plasmid for wild-type renin (pSVDPRnPA33), a mutant renin (pAD317SV) or negative control (pSVΔRn) and cultured at 37°C for 72 h. Conditioned medium of the transfected cells (COS-CM) was assayed for renin activity using hog angiotensinogen as substrate (table 1). COS-CM from the cells transfected with the wild-type or mutant plasmid showed a very low level of activity without activation. However, with trypsinization under mild conditions renin activity increased about 50-fold. A similar level of activity was detected when the COS-CM was dialysed against acidic buffer. The acid activation was a reversible process, because the observed activity disappeared by dialysis against neutral buffer and recovered by redialysis against acidic buffer (not shown). This is characteristic for human prorenin found in human plasma or expressed in CHO cells [21]. Renin activity in the activated COS-CM from the cells transfected with the wild-type plasmid was about 3-fold higher than that of mutant plasmid (table 1). Cells transfected with pSVΔRn, a plasmid lacking most of the renin coding sequence, did not express renin activity (table 1).

Western blot analysis (fig.2) showed that COS-CM from the cells transfected with the wild-type (lane 3) or mutant (lane 5) plasmid gave a single protein band specific for anti-renin antiserum with a molecular mass of 46 kDa, which was in good agreement with the reported value for human prorenin [21]. Digestion with trypsin decreased the size of the protein to 43 kDa (lanes 2,4), a similar value to that observed in human mature renin [22]. These results indicate that the transfected COS cells produced prorenin which was converted to mature renin with trypsin, as reported for transfected CHO cells [15,21].

Activity of wild-type or mutant renin expressed in trypsinized COS-CM was measured at various pH values to evaluate the effect of the Ala to Asp conversion at the 317 residue on the pH-dependence of enzyme activity. Using hog angiotensinogen as substrate, the pH-dependence profile of wild-type renin activity was identical to that of human renal renin (fig.3). In contrast, the optimum pH of mutant renin definitely shifted by 0.5 unit to the acidic side. This result indicated that wild-type renin expressed in COS cells was essentially identical to human renal renin in pH dependency and the substitution of Ala-317 with

| | 317 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human renin | G | P | T | W | A | L | G | [A] | T | F | I | R | K | F | Y |
| Mouse renin | G | P | V | W | V | L | G | [A] | T | F | I | R | K | F | Y |
| Rat renin | G | P | V | W | V | L | G | [A] | T | F | I | R | K | F | Y |
| Human pepsin | G | E | L | W | I | L | G | [D] | V | F | I | R | Q | Y | F |
| Porcine pepsin | G | E | L | W | I | L | G | [D] | V | F | I | R | Q | Y | Y |
| Penicillopepsin | G | - | F | S | I | F | G | [D] | I | F | L | K | S | Q | Y |
| Endothiapepsin | G | - | L | L | I | F | G | [D] | I | F | I | K | A | S | Y |
| Human cathepsin D | G | P | L | W | I | L | G | [D] | V | F | I | G | R | Y | Y |
| Porcine cathepsin D | G | P | L | W | I | L | G | [D] | V | F | I | G | R | Y | Y |
| Bovine chymosin | - | Q | K | W | I | L | G | [D] | V | F | I | R | E | Y | Y |

Fig.1. Comparison of amino acid sequences of renins and other aspartyl proteases. Partial amino acid sequences containing Ala-317 of human [3], mouse [4] and rat [5] renin were aligned to the corresponding sequence of human pepsin [6], porcine pepsin [7], penicillopepsin [8], endothiapepsin [9], human cathepsin D [11] and bovine chymosin [12]. The numbering is based on the human mature renin sequence. Dashes show gaps introduced to obtain maximal homology. The amino acid residues corresponding to Ala-317 of human renin are enclosed.

Table 1

Renin activity in conditioned medium of the transfected COS cells

| Transfected plasmid | Renin activity (ng AI/ml per h) | | |
|---|---|---|---|
| | Untreated | Trypsin-activated | Acid-activated |
| pSVDPRnPA33 (wild-type) | 1.4 | 71.4 | 82.9 |
| pAD317SV (Asp-317 mutant) | <0.5 | 24.9 | 28.4 |
| pSVΔRn (negative control) | <0.5 | <0.5 | ND |

Conditioned medium of COS cells transfected with each plasmid was assayed for renin activity before and after activation. Pro-renin was activated by trypsin in the concentration of 10 μg/ml at 0°C for 30 min or by dialysis at 4°C for 18 h against 0.1 M citric acid/0.2 M $Na_2HPO_4$ (pH 3.4). Renin activity was measured at pH 6.5 using hog angiotensinogen as substrate. ND, not determined

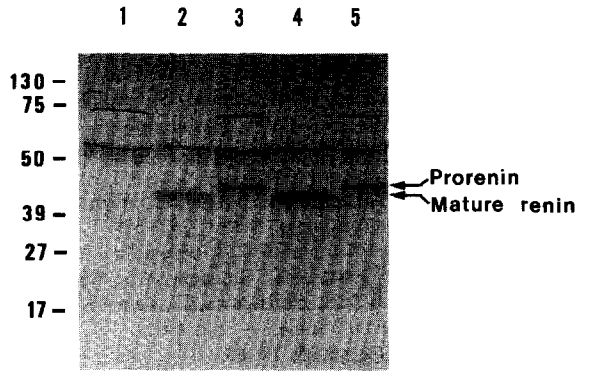


Fig.2. Western blot analysis of proteins expressed in COS-CM. COS-CM from the cells transfected with pSVDPRnPA33 (lanes 2,3), pAD317SV (lanes 4,5) or pSVΔRn (lane 1) was concentrated 24-fold by ultrafiltration. The COS-CMs were subjected to SDS-PAGE before (lanes 1,3,5) or after (lanes 2,4) incubation with trypsin. Proteins were transferred to nitrocellulose filters, incubated with anti-renin antiserum, and developed with horseradish peroxidase.

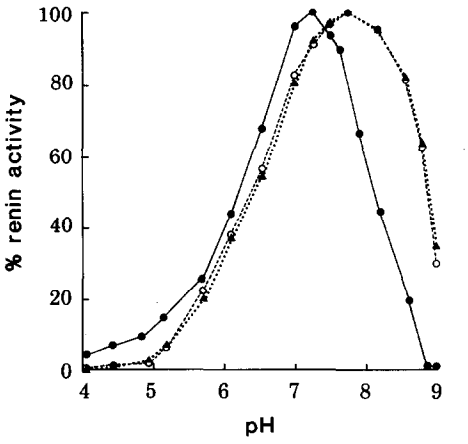


Fig.3. pH-dependence of renin activity with hog angiotensinogen. Activity of wild-type (o---o) or mutant (●—●) renin was measured at the indicated pH using partially purified hog angiotensinogen. Human renal renin (▲---▲) was used as a control. Buffers used were: 0.1 M citric acid/0.2 M $Na_2HPO_4$ for pH 4.0–6.5, 0.2 M sodium phosphate for pH 6.0–8.0 and 0.2 M Tris-HCl for pH 7.5–9.0.

Asp lowered the optimum pH of the enzyme. The observed pH shift to acidic side is probably due to the negative charge of Asp-317 introduced into mutant renin, which can interact directly or indirectly with carboxyl group(s) of catalytically active aspartic acid residues. This 0.5 unit of pH shift is definite but smaller than expected. There may be two explanations about this smaller pH shift. One explanation is that (an)other amino acid residue(s) of renin which is (are) not examined in this study, may also contribute to its neutral optimum pH. Another possibility is that the optimum pH of another aspartyl protease such as pepsin and cathepsin D on angiotensinogen may be more neutral than we assumed. Although direct evidence is not available in the present study, the latter possibility may be assumed by the previous results that the optimum pH values of aspartyl proteases depend on substrate [2,23]. Further studies are needed to clarify the reason why renin has a neutral optimum pH.

In addition to the pH shift, mutant renin had a lower specific activity. The specific activity of wild-type and mutant renin in trypsinized COS-CMs was 13.9 and 7.1 mg AI/h per mg renin, respectively, using a direct radioimmunoassay to determine the renin concentration. However, this result must be confirmed on purified mutant renin expressed in other systems such as CHO cells which are favorable for obtaining milligram quantities of protein.

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