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FLUORIMETRIC ASSAY OF RENIN

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Summary

A simple fluorimetric assay was set up to test renin within 2 h. *N*-acetyl-tetradecapeptide was synthesized and used as substrate. It was demonstrated that *N*-acetyl-angiotensin I and Leu-Val-Tyr-Ser were the two peptides obtained after hydrolysis by renin. Fluorescamine reacted with the free NH_2 of the tetrapeptide generated to induce a fluorimetric reaction detected at 395—495 nm. The Michaelis constant of the reaction was $1.87 \cdot 10^{-5}$ M. With this method as little as one milliGoldblatt Unit (mG.U.) of hog renin could be detected and the generation of tetrapeptide was linear with respect to the renin concentration up to 20 mG.U. The fluorimetric assay was applied to the detection of renin during its purification and to the characterization of renin inhibitors.

Since the first biological assay of renin was developed by Haas and Goldblatt [1], many techniques have been introduced which measure this enzyme. Definite improvement was obtained when it became possible to bioassay not the enzyme itself but the angiotensin generated during the incubation of renin with its substrate under controlled conditions as in Boucher's method [2]. A new impulse was given to the field of renin-angiotensin biochemistry when the radioimmunoassay of angiotensin I became available [3—5]; it was then possible to perform multiple reproducible assays. However, the radioimmunoassay still presents some disadvantages: the necessity to prepare [^{125}I]-angiotensin I and a substrate free from renin; duration of the immunological reaction, which requires 24—48 h; possible interference with non-angiotensin I cross-reacting substances; and influence of angiotensinase inhibitors on the generation of angiotensin I [6,7].

These reasons prompted us to take advantage of the ability of renin to split the tetradecapeptide substrate at the Leu-Leu bond to generate angiotensin I

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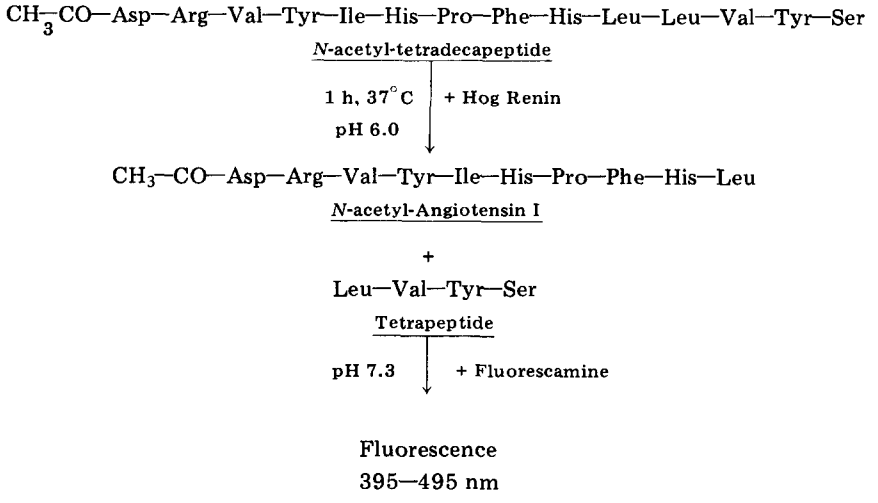
and the tetrapeptide Leu-Val-Tyr-Ser [8,9]. The tetrapeptide was measured by a fluorimetric assay specific for the N-terminal amino groups [10]. To avoid high blank values due to the presence of a free terminal amino group in the commercially available tetradecapeptide, the terminal amino group of the peptide was acetylated and the *N*-acetyl-tetradecapeptide used as a substrate according to the following reaction scheme:

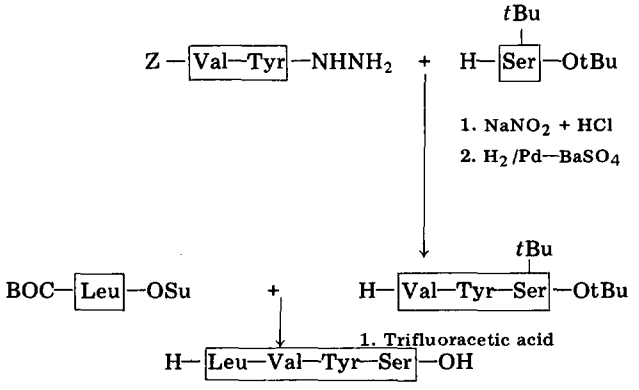


The purpose of this study was to establish a practical renin assay, to characterize the reaction between hog renin and the *N*-acetyl-tetradecapeptide, and to explore the possible applications of this technique to the biochemistry of the renin-angiotensin system.

Materials and Methods

Material

(1) Peptide synthesis. (a) L-Leucyl-L-Valyl-L-Tyrosyl-L-Serine. The synthesis is carried out as illustrated on the following reaction scheme:



Abbreviations are used according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [11]: Z = benzyloxycarbonyl; *t*Bu = tertiary butyl; BOC = tertiary butyloxycarbonyl; Su = *N*-hydroxy-succinimidyl. Yields varied from 60—90%. All intermediates were purified by crystallisation and identified by thin layer chromatography on silica gel. Reagents used were ninhydrin, Pauly and Greig-Liebeck [12].

(b) α-*N*-acetyl-angiotensin I and α-*N*-acetyl-tetradecapeptide *. The free decapeptide and tetradecapeptide were acetylated at pH 6.5 with *p*-nitro-phenylacetate in dimethyl-formamide/H_2O overnight at 25° C. The solvent was evaporated and crude material purified on a Craig-countercurrent distribution apparatus in the system *n*-butanol/acetic acic/water (5 : 1 : 4, v/v). All three peptides were homogenous on thin layer chromatography in the following systems: *n*-butanol/acetic acid/H_2O, 4 : 1 : 1, v/v; *n*-butanol/acetic acid/H_2O/pyridine, 15 : 3 : 12 : 10, v/v; butan-2-ol/H_2O/pyridine, 20 : 11 : 10, v/v.

The analysis of the 3 peptides gave: Leu-Val-Tyr-Ser: $C_{23}H_{36}N_4O_7$ = 480.57; theoretical: C = 57.49%, H = 7.58%, N = 11.66%; Experimental: C = 57.26%, H = 7.60%, N = 11.47%.

N-acetyl-angiotensin I: $C_{64}H_{91}N_{17}O_{15}$ = 1398.54; Theoretical: C = 57.43%, H = 6.58%, N = 17.79%; Experimental: C = 57.25%, H = 6.90%, N = 17.61%.

N-acetyl-tetradecapeptide: $C_{87}H_{125}N_{21}O_{21}$ = 1809.09; Theoretical: C = 58.02%, H = 6.99%, N = 16.33%, Experimental: C = 57.93%, H = 7.04%, N = 16.21%.

(2) Pure hog renin. Renal hog renin (EC 3.4.99.19) prepared by affinity chromatography [13] was further purified by gel filtration and isoelectric focusing. The specific activity of the enzyme was 1100 Glodblatt Units/mg of protein [14]. One Goldblatt Unit was defined as previously reported [15]. The criteria for purity of the renin preparation were: (a) a single band in SDS poly-acrylamide gel electrophoresis and in polyacrylamide gel electrophoresis at pH 7.5; (b) a single precipitin line in double immunodiffusion.

(3) Reagents. Fluorescamine (Fluram) was obtained from Roche. Ultrogel was purchased from LKB. All solvents were analytical grade quality (Prolabo). Pepstatin was most kindly provided by Dr. Umezawa and Aoyagi. Standard hog renin (65/119) was donated by the Medical Research Council, Holly Hill, London.

Methods

(1) Renin assay by fluorimetry. (a) Method. Hog renin samples (from 2 to 20 mG.U.) were allowed to react with 28 nmol of *N*-acetyl-tetradecapeptide for 1 h at 37° C in 1 ml 20 mM citrate/phosphate buffer (pH 6.0). The reaction was stopped by immersion in a boiling water bath and the pH raised to 7.3 by addition of 1 ml 0.1 M citrate-phosphate buffer (pH 7.3). The fluorimetric reaction was developed at room temperature with 0.2 ml Fluram solution (prepared daily by dissolving 30 mg in 100 ml dioxane). The fluorescence was detected at 395—495 nm in a Jobin-Yvon spectrophotofluorimeter. In control experiments, the blank value (of non-incubated medium) was always less than

* This peptide is now commercially available from Bachem, Hauptstrasse 144, CH-4416 Bubendorf (Switzerland).

10% of the incubated mixture. Results were expressed either in relative fluorescence or in nmol of tetrapeptide generated per hour of incubation. A standard curve of tetrapeptide (1—4 nmol) was made during the same experiment.

(b) Characterization of the incubation products. 0.3 G.U. pure hog renin were incubated with 56 nmol of *N*-acetyl-tetradecapeptide for 3 h at pH 6.0. Aliquots were collected every 30 min and tested by radioimmunoassay of angiotensin I [16] and fluorimetric assay. Results were expressed in nmol of peptide generated.

In addition, the two peptides generated were submitted to thin layer chromatography on silica gel. 3 solvent systems were used: *n* butanol/pyridine/acetic acid/water, 30 : 20 : 6 : 24, v/v; *n* butanol/ethyl acetate/acetic acid/water, 1 : 1 : 1 : 1, v/v; $CHCl_3/CH_3OH + NH_3$ 1 : 1, v/v.

(c) Determination of optimal pH. 10 mG.U. pure renin were incubated with $2.8 \cdot 10^{-8}$ mol *N*-acetyl-tetradecapeptide in 1 ml 20 mM citrate phosphate buffer (pH 4.20—pH 7.05). 4 experiments were run at each pH and the relative fluorescence was measured.

(d) Michaelis constant determination. 10 mG.U. of pure renin were incubated with increasing concentrations of *N*-acetyl-tetradecapeptide ranging from 2.8 to 56 μM at pH 6.0. Results were plotted in Lineweaver-Burk form [17] using the least squares method.

(e) Renin assay. Increasing amounts of hog renin (from 2 to 30 mG.U.) were incubated with $2.8 \cdot 10^{-5}$ M *N*-acetyl-tetradecapeptide at pH 6.0. 4 incubations were performed for each renin concentration; results are expressed in nmol of tetrapeptide generated per h.

Renin activity was calibrated using both the fluorimetric and radioimmunological assays with the standard renin preparation. For the fluorimetric assay the test was performed on 10 mG.U. of renin. For the radioimmunoassay, 25 μG.U. renin were incubated for 15 min at pH 6.5 with 250 pmol angiotensinogen, prepared from nephrectomized-rat plasma. Results are expressed in nmol of peptide generated per h by 1 Goldblatt Unit of enzyme.

(2) Application of the fluorimetric renin assay. (a) Detection of hog renin eluted by gel filtration. 6000 G.U. semi-purified hog renin (specific activity 12 G.U./mg protein) were applied to a 5 × 82.5 cm acrylamide-Agarose (Ultrogel AcA 44) column and eluted upwards in 100 mM phosphate buffer (pH 6.5) with a constant flow rate of 1.15 ml/min. 9.5 ml fractions were collected and renin was measured in each fraction by both radioimmunoassay and fluorimetric assay.

(b) Characterization of renin inhibitors. 10 mG.U. of hog renin were incubated at pH 6.0 for 1 h at 37° C with increasing amounts of Pepstatin (0, 1, 2 and $3 \cdot 10^{-7}$ M). 3 different concentrations of *N*-acetyl-tetradecapeptide (5.25, 10.5 and 28 μM) were used. Four determinations were run for each point, and results were plotted according to the Dixon representation [18].

Results

(1) Fluorimetric renin assay

(a) Characterization of the incubation products. Fig. 1 represents the genera-

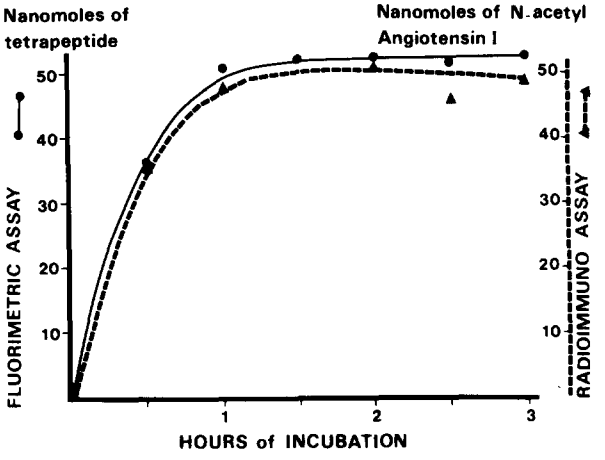


Fig. 1. Generation of the two peptides obtained during the incubation of 56 nmol of *N*-acetyl-tetradecapeptide with 0.3 G.U. hog renin. *N*-acetyl-angiotensin I is measured by radioimmunoassay (▲- - - - -▲) and tetrapeptide by fluorimetric assay (●———●).

tion of peptides obtained during the incubation of *N*-acetyl-tetradecapeptide with an excess of renin: the generation of *N*-acetyl-angiotensin I is measured by radioimmunoassay and the generation of tetrapeptide by fluorimetric assay. The same pattern is obtained for the generation of the two peptides. The plateau observed after 1 h of incubation corresponds to a total hydrolysis of the substrate. The two peptides generated during the incubation had the same Rf as standard *N*-acetyl-Angiotensin I and tetrapeptide in the 3 thin layer chromatography systems described in Methods.

(b) Optimum reaction pH. Fig. 2 represents the fluorimetric response obtained at various pH values. There was no reaction at pH 4.20, while maximum activity was found between pH 5.8 and 6.4. Therefore, pH 6.0 was selected for the fluorimetric assay.

(c) K_m determination. The K_m of *N*-acetyl-tetradecapeptide determined according to the Lineweaver-Burk plot is shown in Fig. 3. A K_m of $1.78 \cdot 10^{-5}$ was obtained at pH 6.0.

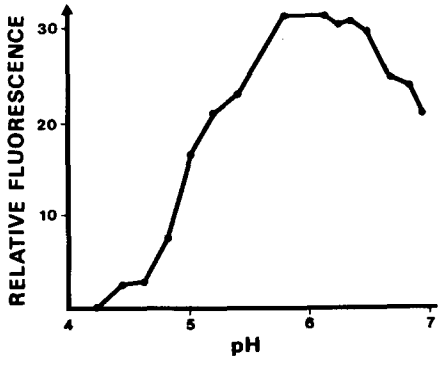


Fig. 2. Hydrolysis of *N*-acetyl-tetradecapeptide by hog renin at pH 4.0—7.0.

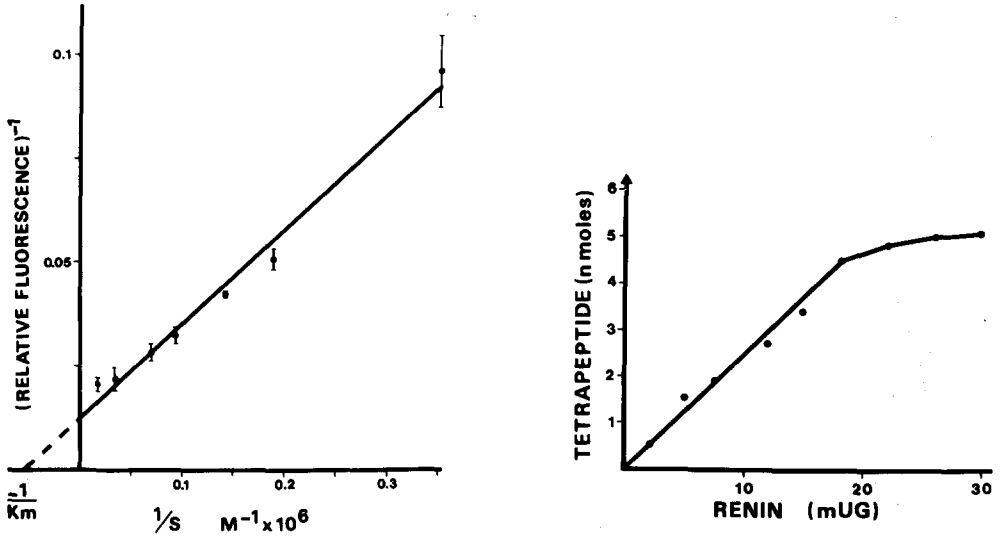


Fig. 3. Lineweaver-Burk plot of the reaction between *N*-acetyl-tetradecapeptide and hog resin. Abscissa: reciprocal plot of increasing concentrations of substrate. Ordinate: reciprocal plot of relative fluorescence obtained in the incubation. The brackets represent the mean ± S.E.M.

Fig. 4. Generation of tetrapeptide for increasing concentrations of renin (2—30 mG.U.). The substrate concentration is 28 μM. Each point represents the mean of 4 determinations.

(d) Renin concentration measurement. Fig. 4 demonstrates that the reaction was linear with respect to the renin concentration under 20 mG.U. Beyond this concentration the rate of tetrapeptide generation decreases suggesting that the substrate was no longer in excess. From the linear part of the curve it was calculated that 1 Goldblatt Unit of renin generated 187 nmol product (tetrapeptide or angiotensin I) per h. Under the radioimmunoassay conditions 62 nmol angiotensin I were generated per h by 1 Goldblatt unit. The intra- and inter-assay coefficients of variation were respectively 11.3% (n = 10) and 19.5% (n = 10).

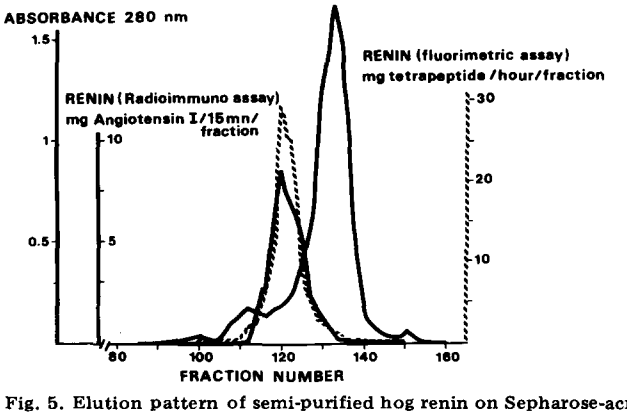


Fig. 5. Elution pattern of semi-purified hog renin on Sepharose-acrylamide gel. Proteins are determined at 280 nm (———). Renin is measured by two methods: radioimmunoassay (———) and fluorimetric assay (- - - - - -).

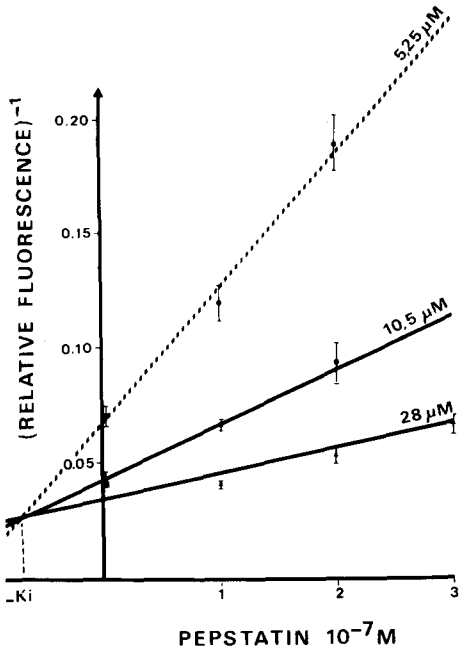


Fig. 6. Dixon plot of the inhibition of hog renin by pepstatin. The concentrations of *N*-acetyl-tetradecapeptide are 5.25, 10.5 and 28 μM. Abscissa: concentrations of pepstatin. Ordinate: reciprocal plot of the relative fluorescence.

(2) Application of the fluorimetric assay

(a) Measurement of renin eluted by gel filtration. Renin was detected in the same fractions using the two methods of measurement: radioimmunoassay or fluorimetric assay. The same elution pattern was obtained for the enzyme and the maximum activity was found in the same fraction (Fig. 5). No extra peak of renin was detectable when either the fluorimetric or the radioimmunological assays were used.

(b) Characterization of inhibition by Pepstatin. Fig. 6 shows the characteristics of pepstatin inhibition: pepstatin behaves as a competitive inhibitor with K_i $0.7 \cdot 10^{-7}$ M.

Discussion

Several methods have already been described concerning the chemical or radiochemical assays of renin on synthetic substrates: Reinhartz and Roth [19] were able to test human and hog renin using Z-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-naphthylamide as substrates. After cleavage of the Leu-Val-Tyr-Ser-β-naphthylamide peptide, the aminopeptidase M was used to permit a fluorimetric assay. The fluorescence of the naphthylamine generated was directly related to the concentration of renin. However, this method presents two disadvantages: (1) two reactions are needed; (2) it requires the mediation of an auxiliary enzyme, aminopeptidase M, in order to generate the naphthylamine and the commercially available aminopeptidase M is not always devoid

of endopeptidasic activity. Levine et al. [20] worked with the nonapeptide substrate His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser. The tetrapeptide obtained after incubation with renin was separated from the substrate by Technicon Dialysis and measured by the Folin-Ciocalteu reagent. The lack of assay sensitivity required the presence of a large amount of substrate which made the method very expensive.

The radiochemical assays of renin use labelled substrates. The peptide generated during the incubation has to be separated from the substrate: Mendelsohn [21] prepared a [^{14}C]valine-labelled tetradecapeptide and isolated the labelled angiotensin I by paper chromatography; Ontjes et al. [22] synthesized a heptadecapeptide substrate with a [^{3}H]leucine at the 10 position. This peptide was covalently attached to Sepharose and the labelled angiotensin I was obtained by filtration. Bath and Gregerman [23] coupled an iodinated tridecapeptide to the polyglutamic acid; the ^{125}I-labelled tetrapeptide generated was extracted in butanol. More recently several authors have set up renin assays based on the use of labelled natural angiotensinogen [24,25], but this technique requires a completely pure angiotensinogen.

In the method described here the substrate can be easily obtained and only 50 μg are necessary per assay. There is no reaction of the substrate with the fluorescamine; thus, the tetrapeptide can be detected directly in the incubation mixture. It has been verified that the affinity of hog renin for *N*-acetyl-tetradecapeptide ($K_m = 1.8 \cdot 10^{-5}$ M) and the tetradecapeptide ($K_m = 0.77 \cdot 10^{-5}$ M) were quite similar. Under these conditions, as low an 1 mG.U. of hog renin could be detected and the linearity of the reaction was observed up to 20 mG.U. Sensitivity of the reaction can be compared with the results obtained by others: Levine et al., 100 mG.U./ml; Reinhartz, 10 mG.U.; Mendelsohn, 10 mG.U.

The validation of this technique has been established with pure hog renin as a source of enzyme. α-*N*-acetyl-angiotensin I and tetrapeptide have been proved to be the products of the renin reaction by radioimmunoassay and thin layer chromatography. The fluorimetric assay is not restricted to the enzymatic studies on pure renin only. It is routinely used in our laboratory to detect hog renin in semi-purified preparations. However, great care should be taken before applying the technique to crude renin since contaminating proteases could interfere in the assay by generating free NH_2 from other peptides.

Another field of application was the detection of inhibitor(s) acting on the renin substrate reaction in the angiotensin I radioimmunoassay. We had previously demonstrated that pepstatin was a competitive inhibitor of the reaction between hog renin and rat angiotensinogen ($K_i = 0.75 \cdot 10^{-7}$ M) [26]. Using *N*-acetyl-tetradecapeptide as renin substrate and the fluorimetric assay, we still found that pepstatin is a competitive inhibitor with a K_i of $0.7 \cdot 10^{-7}$ M. Therefore this method could be useful as a screening test for other renin inhibitors.

In conclusion, the fluorimetric assay of renin is a sensitive and time saving method for measuring this enzyme. It can replace the radioimmunoassay in many circumstances and provides a new, simple tool for the biochemical study of the renin-angiotensin system.

Acknowledgments

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