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Affinity Chromatography of Renin Using Inhibitory Renin Substrate Analogs[1] (38170)

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(Introduced by W. E. Lassiter)

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Inhibitors of the renin-angiotensin system may interfere at any one of several steps between the release of renin from the kidney and the final action of angiotensin II upon target tissues. Kokubu and co-workers have reported that certain small peptides, particularly the methyl esters of Leu-Leu-Val-Tyr and Leu-Leu-Val-Phe, are direct inhibitors of the action of renin upon its plasma substrate, angiotensinogen (1). These inhibitory peptides are analogs of the amino acid sequence adjacent to the leucyl-leucine bond, which is known to be cleaved by renin. We report here our characterization of the inhibitory properties of several small peptide analogs of the renin substrate, and the use of such peptides as agents for the reversible binding of hog and human renin in affinity chromatography.

Materials and Methods. All peptides were synthesized by standard procedures using α-*t*-butyloxycarbonyl-L-amino acids (Bachem, Marina Del Ray, California) and coupling with dicyclohexylcarbodiimide (2). The benzyl ether group was used for blocking the hydroxyl function of tyrosine, and methyl esters were used for blocking the carboxyl function of the carboxyl terminal amino acid in each peptide. α-Amino blocking groups were removed by treatment for 30 min with 4 *N* HCl in dioxane, while final deblocking of tyrosine peptides utilized anhydrous HF for 1 hr at 0° for removal of benzyl ethers (3). Crude peptides were purified during the course of synthesis by recrystallization from methanol–ether and in some cases by partition column chromatography on Sephadex LH-20 in methanol–ethyl acetate (1:2). The purified peptides were homogeneous as judged by thin layer chromatography on silica gel plates (Brinkman F-254) in butanol–acetic acid–water (4:1:5 v/v, upper phase) and methanol–ethyl acetate (1:2 v/v). The experimentally determined amino acid composition of each peptide was within 10% of theoretical values.

The peptide analogs were coupled to agarose via their free α-amino groups according to methods described by Cuatrecasas (4, 5). Two different derivatives of agarose (Sepharose 4B, Pharmacia Co.) were prepared for use in peptide coupling. The first derivative, succinylated aminoethyl agarose, was obtained by coupling ethylene diamine to Sepharose with the use of cyanogen bromide. This product was reacted with succinic anhydride to yield a derivative with a free carboxyl group. The second derivative, succinylated diaminodipropylamino agarose, was prepared in the same way as the first derivative, except that 3,3′-diaminodipropylamine was used as the amine component in the cyanogen bromide coupling reaction (4). To couple the peptide analogs, 0.5 mmole of the desired peptide was stirred with 20 ml bed volume of

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derivatized agarose and 30 ml water–dimethylformamide (1:1) at pH 5. After addition of 1.25 mmoles 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Eastman Chemical Co.), the mixture was stirred at 25° for 18 hr, and then rinsed exhaustively with water–dimethylformamide (1:1), 0.5 *M* acetic acid, and water. Peptide content, as determined by amino acid analysis of an acid hydrolysate of each agarose derivative, was adjusted to 0.2 to 0.3 μ-moles per ml bed volume by dilution with untreated agarose.

For the experiments with human renin, additional peptide-agarose derivatives were prepared by coupling peptides via the active *N*-hydroxysuccinimide ester of succinylated diaminodipropylamino agarose (5). In this case a measured bed volume of the freshly prepared active ester derivative was stirred at 5° for 12 hr with an equal volume of 0.1 *M* Na_2HPO_4, 5% ethylene glycol, pH 6.5, containing the desired peptide analog at a concentration of 10 m*M*. The coupling reaction was terminated by addition of excess glycine with stirring for 2 hr at 25°. After prolonged rinsing with 0.1 *M* Na_2HPO_4 buffer, 0.5 acetic acid, and water the peptide content was determined as above.

Partially purified hog kidney renin (0.7–0.8 Goldblatt units/mg, Miles Laboratories) and a standardized preparation of human kidney renin (0.13 units/mg, a gift of Dr. Erwin Haas) were used in all experiments. Two independent *in vitro* renin assay procedures were used to test for inhibitory activity. The first assay, a radiochemical procedure developed in this laboratory (6), measured the rate of cleavage of a tritiated derivative of angiotensin I from a synthetic analog of the tetradecapeptide fragment of natural renin substrate. The substrate peptide had been insolubilized by coupling to agarose via its carboxyl terminus as previously described. This assay was used routinely for both hog and human renin experiments. A second assay was performed by the radioimmunoassay of angiotensin I (7), produced during incubation of pooled nephrectomized human plasma with varying amounts of added human renin. Rabbit antiserum against angiotensin I, and ^{125}I-angiotensin I were purchased from New England Nuclear Corp. In both assays, the rate of substrate cleavage was directly proportional to the experimental concentrations of added renin.

Results. Inhibitory properties of peptides in solution. Each peptide analog was tested for inhibitory activity at concentrations ranging from 10^{-6} *M* up to its limits of solubility, usually 10^{-3} *M*. The radiochemical assay was used to measure inhibition of both hog and human renins, while the angiotensin I immunoassay was used to measure inhibition of the action of human renin against natural human substrate. In both the radiochemical assay and the immunoassay, the activity observed at each concentration of peptide inhibitor was expressed as a percentage of the activity seen in the absence of an inhibitor. From graphs of renin activity versus inhibitor concentration for each peptide (not shown) we estimated the peptide concentrations required to produce a 50% inhibition in both assay systems (Table I). The results for both hog and human renin in the radiochemical assay were similar. The peptides Leu-Leu-Val-Tyr-OCH_3 and Leu-Leu-Val-Phe-OCH_3 were the best inhibitors; Leu-Val-Phe-OCH_3 was less active; and Leu-Leu-Val-OCH_3, Leu-Leu-Val-Gly-OCH_3, and Leu-Val-OCH_3 were inactive at the concentrations tested. In the immunoassay system using natural human plasma substrate, higher concentrations of the active peptides were required to achieve 50% inhibition. Thus, the peptides were less effective in competing against the natural substrate than against the insolubilized substrate.

The results of a kinetic study of the action of the prototype inhibitor Leu-Leu-Val-Phe-OCH_3 upon hog renin are shown in Fig. 1, in which concentrations of the radiolabeled synthetic substrate were varied over a sixfold range. The inhibition was of the classical competitive type. The calculated inhibitory constant, K_i (8), was 180 μM, while the calculated K_m for the insolubilized synthetic substrate was 12.5 μM. Although Leu-Leu-Val-Phe-OCH_3 acted as a competitive inhibitor of renin, no evidence of

TABLE I. Peptide Concentrations Required for 50% Inhibition of Activity.

Peptide	Peptide concentration (m*M*) [a]		
	Hog renin with synthetic substrate[b]	Human renin with synthetic substrate[b]	Human renin with plasma substrate[c]
Leu-Leu-Val-Tyr-OCH_3	0.32	0.05	1.2
Leu-Leu-Val-Phe-OCH_3	0.35	0.18	2.0
Leu-Val-Phe-OCH_3	1.0	0.44	3.0
Leu-Leu-Val-OCH_3	>1.0	>1.0	>3.0
Leu-Leu-Val-Gly-OCH_3	>1.0	>1.0	>3.0
Leu-Val-OCH_3	>1.0	>1.0	>3.0

[a] The values are means from 2 separate experiments, with 50% inhibitory concentrations derived as described in the text.

[b] Incubated in 0.1 *M* citric acid, 0.2 *M* Na_2HPO_4, 1 m*M* EDTA, pH 6.5 for 2 hr at 37° (6).

[c] Incubated for 2 hr at 37° after first diluting human nephrectomized plasma 1:3 with 0.2 *M* Na malcate, pH 6.0.

cleavage of the leucyl-leucine bond of the inhibitor could be found. A 1 m*M* solution of the inhibitor was incubated in 0.1 *M* citric acid, 0.2 *M* Na_2HPO_4 buffer, pH 6.5, with 0.1 units/ml of human or hog renin for 24 hr at 37°. After the incubation no free leucine could be detected, either by amino acid analysis or by thin layer chromatography on silica gel plates in butanol–acetic acid–water. We had previously found that the peptide *N*-acetyl-[^{3}H] Leu-Leu-Val-Tyr-Ser-Gly-Lys-Pro-OH was also not cleaved by renin (6).

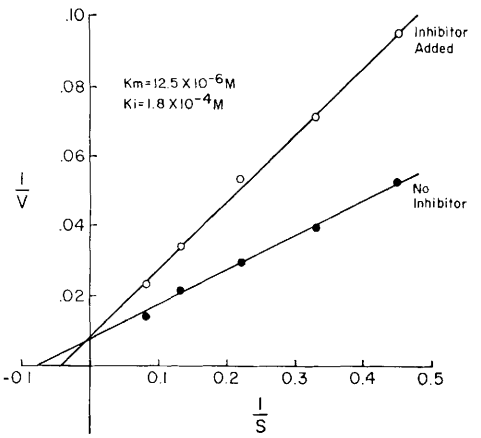

FIG. 1. Reciprocal plot of the cleavage of synthetic substrate by hog renin in the presence or absence of the inhibitor Leu-Leu-Val-Phe-OCH_3, at a concentration of 200 μM. Substrate concentration is expressed in μmoles and velocity in pmoles angiotensin I generated/min/mg hog renin, as previously described (6).

Binding of renin to peptide-agarose conjugates. To test directly for the binding of renin to the various inhibitor analogs, we prepared chromatographic columns of peptide-agarose conjugates and applied 5 mg samples of crude hog or human renin. Column eluates were monitored for renin activity by the radiochemical assay and for protein concentration (9). The behavior of hog renin in eluting from four different peptide-agarose columns is shown in Fig. 2. Each column had been prepared by coupling the peptide to succinylated aminoethyl agarose by the carbodiimide procedure. In the columns containing the active inhibitors Leu-Val-Phe-OCH_3 and Leu-Leu-Val-Phe-OCH_3, inactive protein was eluted rapidly with the void volume, while renin activity was delayed and separated from the bulk of the protein. With the stronger inhibitor, Leu-Leu-Val-Phe-OCH_3, renin activity was not recovered until after the pH of the eluting buffer was changed from 6.5 to 3.0. A purification of approximately 25 fold was achieved, based on measurements of activity and protein concentration. With the weaker inhibitor, Leu-Val-Phe-OCH_3, the activity was eluted before the change in buffer pH. When the inactive analogs Leu-Leu-Val-OCH_3 and Val-Phe-OCH_3 were present on the columns, there was no evidence of any binding phenomenon. Both protein and

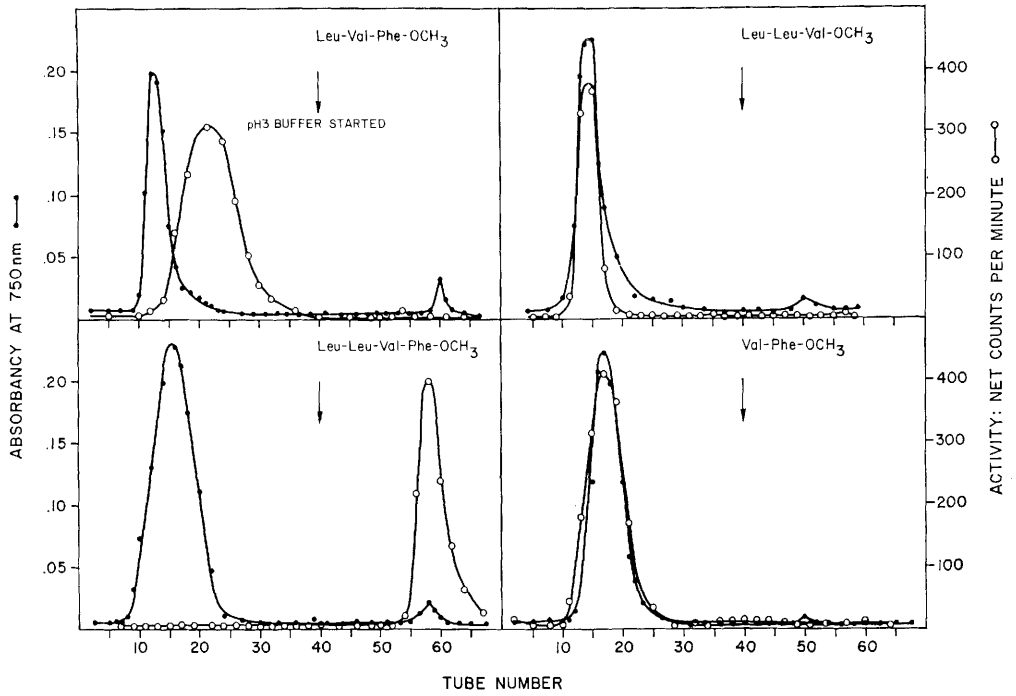

FIG. 2. Chromatography of hog renin on four different peptide-agarose columns. The peptide content of each 1 × 15 cm column was between 0.2 and 0.3 μmoles per ml bed volume. Elution was with 0.1 *M* citric acid, 0.2 *M* Na_2HPO_4, 1 m*M* EDTA, pH 6.5, followed by 0.02 *M* acetic acid, pH 3. Fractions were 1.0 ml.

renin activity were eluted together. The recovery of applied hog renin activity was satisfactory for all columns, averaging about 65%.

Human renin behaved differently from hog renin in its interactions with the peptide-agarose conjugates, and greater technical difficulties were encountered in performing satisfactory column experiments. We found that the recovery of applied activity was very poor when columns of succinylated aminoethyl agarose were conjugated with peptides by the carbodiimide procedure. This problem could be overcome by using the *N*-hydroxysuccinimide ester coupling procedure. Unlike hog renin the elution of human renin was not delayed on columns containing active inhibitors coupled to succinylated aminoethyl agarose. However, when succinylated diaminodipropylamino agarose was used as the insoluble support, binding of human renin did occur. The active inhibitor peptides Leu-Leu-Val-Phe-OCH_3 and Leu-Leu-Val-Tyr-OCH_3 were conjugated by the active ester method, and the chromatographic experiments shown in Fig. 3 were performed. With the Leu-Leu-Val-Phe-OCH_3 column there was a very slight delay in the elution of activity, while with the Leu-Leu-Val-Tyr-OCH_3 column the delay was greater. In the latter column the activity peak occurred 12 fractions later than the peak of maximum protein concentrations. In neither column was there a complete separation of renin activity from inactive protein.

Discussion. We have confirmed earlier findings that small substrate analogs may act as competitive inhibitors of renin. Analogs lacking the leucyl-leucine bond were originally reported to be inactive (1). We agree that the presence of the leucyl-leucine bond in the inhibitor sequence enhances inhibitory activity, but it is not an absolute structural requirement, since the peptide Leu-Val-Phe-OCH_3 has slight activity. Analogs lacking an aromatic side chain in the carboxyl-terminal position were uniformly

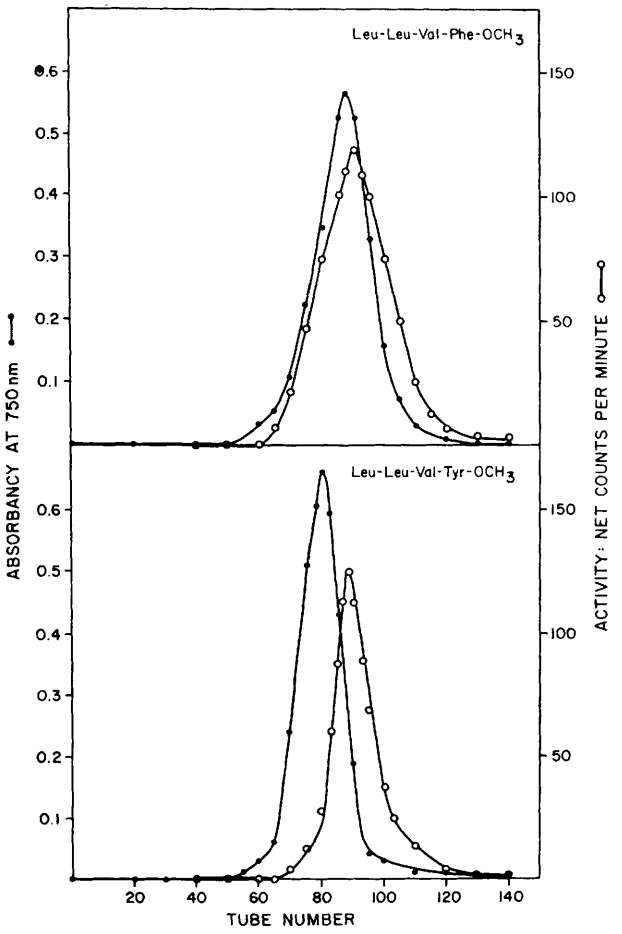

FIG. 3. Chromatography of human renin on two 1.5 × 100 cm agarose columns containing inhibitor peptides coupled by the active ester method. The peptide content of the Leu-Leu-Val-Phe-OCH_3 column was 0.16 μmoles per ml bed volume and the content of the Leu-Leu-Val-Tyr-OCH_3 column was 0.17 μmoles per ml. Elution was with the pH 6.5 buffer.

inactive. In their original report, Kokubu and co-workers assayed for inhibitory activity against rabbit renin, using a rabbit plasma substrate. They found that Leu-Leu-Val-Tyr-OCH_3 and Leu-Leu-Val-Phe-OCH_3 produced a 50% inhibition in concentrations of about 2 mg/ml (2–3 m*M*). Our values for 50% inhibition by the same peptides were quite comparable when the action of human renin against human plasma substrate was being assayed. Smaller concentrations of the inhibitors were effective when hog or human renin was being assayed against the synthetic substrate. In relative terms, the K_i of Leu-Leu-Val-Phe-OCH_3 was 15 times greater than the K_m observed for hog renin with the synthetic substrate, and 50 times greater than the K_m reported by Skeggs (10) for the natural hog substrate. Because of their sparing solubility in water and the rather high concentrations required for inhibition, the analogs described here would be unsuitable for producing *in vivo* effects on the renin-angiotensin system.

A more promising investigative application of the inhibitors appears to be in the affinity chromatography of renin. Our experiments show that renin may be selectively and reversibly bound when exposed to peptide-agarose conjugates under mild conditions. Analogs which were capable of inhibiting renin in solution were also capa-

ble of delaying the elution of renin from an affinity column, while inactive analogs failed to produce such a delay. Obviously, there are additional factors which affect the column separation phenomenon. One of these factors appears to be the length of the bridge or arm which connects the ligand to the insoluble supporting polymer. In our earliest attempts to produce chromatographic reagents by the direct coupling of Leu-Leu-Val-Phe-OCH_3 to agarose, we observed no binding of either hog or human renin. Hog renin could bind when the ligand was attached via a short connecting arm, while human renin could bind only when the arm was lengthened further. It seems likely that these differences between human and hog renin may relate to different steric requirements for the binding of substrates at the catalytic sites.

It has been recently reported that Pepstatin A is an effective ligand for the affinity chromatography of mouse submaxillary gland renin (11) and hog renal renin (12). This pentapeptide, derived from species of *Streptomyces,* is a potent inhibitor not only of renin, but of pepsin and several other acid proteases (13). As one would expect, pepsin as well as renin was retained by the Pepstatin affinity columns (12). In contrast to Pepstatin, peptides such as Leu-Leu-Val-Tyr-OCH_3 are pepsin substrates and are also readily cleaved by pepsin when attached to agarose (6). Since small peptide analogs of renin substrate may be easily and inexpensively synthesized, a larger scale application of these methods would seem to be a promising approach for the purification of renin or the extraction of renin from biological samples.

Summary. Several short peptide analogs of renin substrate were tested for their ability to inhibit renin in solution and to bind renin in affinity chromatography. The peptides Leu-Leu-Val-Tyr-OCH_3, Leu-Leu-Val-Phe-OCH_3 and Leu-Val-Phe-OCH_3 had *in vitro* inhibitory activity against both hog and human renin. Kinetic studies of the prototype inhibitor Leu-Leu-Val-Phe-OCH_3 demonstrated classical competitive inhibition, with a K_i of 180 μM. When impure hog renin was eluted from succinylated aminoethyl agarose columns containing covalently bound Leu-Leu-Val-Phe-OCH_3 or Leu-Val-Phe-OCH_3, the elution of renin activity was delayed in comparison with the elution of inactive contaminating proteins. The binding of human renin to agarose bound inhibitors could also be demonstrated if a longer connecting arm was interposed between the peptide ligand and the supporting polysaccharide.

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