Biochemical Pharmacology, Vol. 30, No. 18, pp. 2630–2631, 1981.
Printed in Great Britain.

0006-2952/81/182630–02 $02.00/0

Lack of inhibition of human renin by human des-angiotensin I renin substrate

(*Received* 28 *October* 1980; *accepted* 12 *March* 1981)

The remnant of renin substrate from which angiotensin I (ANG I) has been released is referred to as des-ANG I renin substrate. Recently Barret *et al.* [1] reported that human des-ANG I renin substrate competitively inhibited the human renin–renin substrate reaction. We have purified the homogeneous human renin substrate from blood bank plasma [2]. Here we report that we prepared human des-ANG I renin substrate with more than 95 per cent purity from the homogeneous human renin substrate and could not observe the inhibitory effect of des-ANG I renin substrate at concentrations up to 1.5 μM on human renin–renin substrate reaction.

Materials and methods

Materials. Ultrogel AcA 44 was purchased from LKB-Produkter, Bromma, Sweden. Dansyl chloride and dansyl amino acids were from the Sigma Chemical Co., St. Louis, MO, U.S.A. Puromycin dihydrochloride was from Makor Chemicals Ltd., Jerusalem, Israel. Standard human renin (Lot No. 68/356) was provided by the National Institute for Biological Standards and Control, Hampstead, London, U.K.

Preparation of human renin. Human kidneys were obtained at post mortem. Human renin that was prepared by the method described previously [2] was further purified by gel filtration on an Ultrogel AcA 44 column (2.5 × 90 cm). The preparation of human renin contained 0.77 Goldblatt units (G.U.)/mg protein when tested *in vitro* against standard human renin.

Preparation of human renin substrate. Homogeneous human renin substrate was prepared from blood bank plasma (2000 ml) by procedures described previously [2]. The purified human renin substrate generated 9.8 μg ANG I/mg protein.

Preparation of human des-ANG I renin substrate. The purified renin substrate (154 nmoles ANG I equivalents) was incubated with human renin (1.85 G.U.) at pH 6.2 and 37° for 20 hr in the presence of 10 mM EDTA, 5 mM 1,10-phenanthroline, and 1 mM puromycin to inhibit aminopeptidase activity. Then the incubation mixture (3.0 ml) was applied to the column of Ultrogel AcA 44 as described above. The column could separate des-ANG I renin substrate from human renin (Fractions No. 90–100). The fractions of a major protein peak (No. 76–88) were pooled and concentrated using an Amicon ultrafiltration apparatus with PM-10 membrane. The concentrated sample that still generated a small amount of ANG I when incubated with renin was again incubated with 1.5 G.U. of human renin for 24 hr under the same conditions. After separating des-ANG I renin substrate from renin by Ultrogel AcA 44 column and concentrating it, the sample was subjected to isoelectric focusing (LKB 110 column) to remove traces of intact renin substrate and contaminating proteins [2]. The fractions (pH 4.3 to 4.7) were pooled and dialyzed against 0.1 M phosphate buffer (pH 7.8). Finally, the sample was applied to the same Ultrogel AcA 44 column to completely remove Ampholine, 69.5 nmoles of des-ANG I renin substrate were obtained.

Renin–renin substrate reaction. One milliliter of the reaction mixture contained human renin (0.01 G.U.), homogeneous human renin substrate (0.15 nmole), various amounts of the purified des-ANG I renin substrate (0 to 1.5 nmoles) and 20 mM phosphate buffer (pH 7.3 or pH 6.0) in the presence of EDTA (10 mM), 8-hydroxyquinoline (3.4 mM), and 2,3-dimercapto-1-propanol (4.8 mM). The reaction mixture was incubated at 37° for 10 min. After boiling it for 5 min to stop the reaction, the ANG I generated was measured by radioimmunoassay for ANG I [3].

Determination of NH_2*-terminal amino acid.* NH_2-terminal amino acids of purified renin substrate and des-ANG I renin substrate were determined by the dansylation technique according to the modified method of Gros and Labouesse [4]. Bovine serum albumin was used as control.

Other methods. Ultrogel AcA 44 gel filtration, isoelectric focusing, and polyacrylamide gel electrophoresis were carried out according to methods described previously [2, 5]. Protein was estimated by the method of Lowry *et al.* [6].

Results and discussion

The preparation of des-ANG I renin substrate resulted in more than 95 per cent purity as indicated by polyacrylamide gel electrophoresis (Fig. 1). The determination of the NH_2-terminal amino acids of the purified renin substrate revealed aspartic acid and small amounts of leucine, valine and alanine, indicating that the renin substrate was contaminated with des-ANG I renin substrate and other proteins. On the other hand, the majority of leucine and small amounts of valine and tyrosine were detected as NH_2-terminal amino acids in the preparation of des-ANG I renin substrate. These results show that the preparation of des-ANG I renin substrate was a mixture of des-ANG I renin substrate and small amounts of proteins that were produced from des-ANG I renin substrate by contaminating aminopeptidase(s) not completely inhibited in the human renin preparation. Degradation products of des-ANG I renin substrate were not distinguishable from des-ANG I renin substrate by gel filtration on Ultrogel AcA 44, preparative isoelectric focusing, and polyacrylamide gel electrophoresis. Tewksbury *et al.* [7] have shown two NH_2-terminal amino acids, aspartic acid and alanine, in their preparations of homogeneous human renin substrate. Valine and tyrosine are constituents of the cleavage product of tetradecapeptide [8].

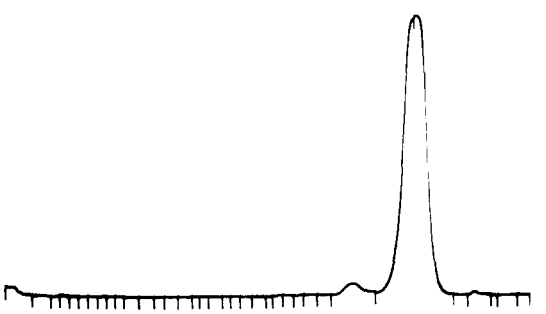

Fig. 1. Polyacrylamide gel electrophoresis of the purified human des-ANG I renin substrate at pH 8.3. The R_f value of the des-ANG I renin substrate was 0.65 when BPB was used as marker.

K_m values of 0.43 μM (homogeneous human renin substrate) at pH 7.3 and of 0.25 μM at pH 6.0 were obtained from Lineweaver–Burk plots at seven different concentrations of renin substrate. Figure 2 shows that des-ANG I renin substrate, over the range of concentrations used, did not affect the human renin-renin substrate reaction at pH 7.3. The same result was obtained when the reaction was carried out at pH 6.0.

Lucas *et al.* [9] have suggested that human plasma protein of the same molecular size as renin substrate (des-ANG I renin substrate?) inhibits human renin. Barret *et al.* [1] have reported that semipurified human des-ANG I renin substrate competitively inhibited human renin–renin substrate reaction. The maximum concentration of des-ANG I renin substrate used in their studies was approximately two times higher than that of renin substrate. In the present study, we used the purified des-ANG I renin substrate, though the preparation contained small amounts of degradation products of des-ANG I renin substrate. The concentrations of des-ANG I renin substrate (up to 10-fold higher than that of renin substrate) might be sufficient to detect the inhibitory effect of des-ANG I renin substrate on the reaction of renin with renin substrate. Hackenthal *et al.* [10] have reported that des-ANG I renin substrate prepared from rat renin substrate had no inhibitory effect on the reaction of partially purified rat renin with rat renin substrate. Scharpé *et al.* [11] have reported that α-1 antitrypsin in human plasma competitively inhibited pig kidney renin. The semipurified des-ANG I renin substrate by Barret *et al.* [1] might have contained sufficient amounts of α-1 antitrypsin that had been concentrated in parallel during partial purification of renin substrate. The discrepancy between the present result and that by Barret *et al.* [1] cannot be explained clearly.

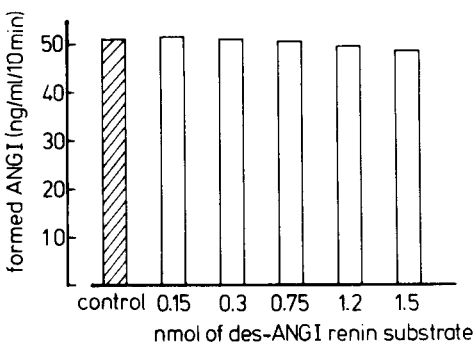

Fig. 2. Effect of various amounts of the purified human des-ANG I renin substrate (0.15 to 1.5 μM) on the reaction of human renin with homogeneous human renin substrate. The incubation conditions were described in the text. Values are means of duplicate determinations.

Acknowledgement—This study was supported in part by a grant from the Kanae Medical Foundation (1977).

The 2nd Department of Internal Medicine
Ehime University School of Medicine
Onsen-gun, Ehime 791–02, *Japan*

KUNIO HIWADA
YUKIMI SOGO
YASUHARU TAKADA
TATSUO KOKUBU*

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* Author to whom all correspondence should be addressed.