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INFLUENCE OF pH ON HUMAN RENIN ACTIVITY WITH DIFFERENT SUBSTRATES: ROLE OF SUBSTRATE DENATURATION

L. FAVRE, R. ROUSSEL-DERUYCK AND M. B. VALLOTTON

Laboratoire d'Investigation clinique, Division d'Endocrinologie, Département de Médecine, Université de Genève, Genève (Switzerland)

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SUMMARY

1. The activity of human renin (EC 3.4.4.15) was studied with four different substrates, two of human origin, one of hog origin and one synthetic substrate.

2. The formation of angiotensin I was determined by radioimmunoassay.

3. A different optimum pH was obtained with each substrate: pH 5.5 with human substrate in plasma, pH 6 with partially purified human substrate, pH 7.5 with hog substrate and pH 4.5 with tetradecapeptide substrate, *i.e.* the N-terminal sequence of the three latter substrates.

4. These different optima in the presence of the same enzyme could be due to a direct effect of pH on the substrate. Such an effect was demonstrated with human substrate by its progressive acidification before incubation at its optimum pH with human renin. Below pH 5 the acidification produced an irreversible inactivation of natural or partially purified human substrate, whereas hog substrate, tetradecapeptide substrate and human renin offered a better resistance to acid pH.

5. When compared to the low optimum pH obtained with tetradecapeptide substrate, the higher optimum of the pH curve of human renin activity with human substrate is thus attributed to an acid denaturation of this substrate.

INTRODUCTION

Several authors have studied the influence of pH on human renin (EC 3.4.4.15) activity with human substrate[1–5] or substrates of other species[6,7]. Different optimum pHs corresponding to each substrate have been reported. Some authors also noted that the substrate itself was affected by acid pH[1,8].

The purpose of the present work was to determine the optimum pH of human renin activity with four substrates, two of them homologous, one heterologous and one of synthetic origin, and to dissociate the respective effects of pH on the enzyme and on the substrates.

For this study purified human renin was successively assayed with natural human substrate from plasma, partially purified human substrate, hog substrate and 1–14 tetradecapeptide substrate. The direct product of these enzymatic reactions, *i.e.* angiotensin I, was measured with a specific radioimmunoassay[9,10], both destruction of the peptide by angiotensinases and conversion to angiotensin II being inhibited*.

MATERIAL AND METHODS

Enzyme

*Human renin***. This was a purified extract from kidneys[11], free of angiotensinases, which was verified not to contain converting enzyme and possessed a specific activity of 0.15 Goldblatt unit (GU) per mg protein. Concentrated solutions of 1 GU per ml were prepared in saline and dilute solutions in 0.1 M Tris–acetate buffer (pH 7.5). The concentrated renin solutions were stable at −20 °C, but repeated thawing and freezing of the dilute solutions led to a progressive loss of enzymatic activity which varied according to their frequence and to the concentration of the solution. Consequently, variations in reproducibility were observed amounting up to 25%. Each complete pH curve was performed with the same renin preparation and each point was done in duplicate. Thus within each pH curve absolute values (as given in the tables) can be compared, while only relative values can be compared between curves (as represented in the figures).

Substrates

Plasma human substrate. This was obtained from human plasma without any further purification. The substrate concentration was indirectly measured as for the other substrates by the maximum amount of angiotensin I generated at optimum pH with an excess of human renin and was expressed in pmoles per ml. The converting enzyme and the angiotensinases present in plasma were inhibited by EDTA and DFP (0.2 ml 0.16 M Na_2-EDTA and 20 μl 1% DFP in 99% isopropanol per 5 ml plasma). The values of endogenous plasma renin activity were negligible compared to those obtained with the added renin; consequently they were not substracted in the final results.

Partially purified human substrate. This was prepared from human plasma according to Skeggs *et al.*[4], and was purified about 4-fold and deprived of renin, converting enzyme and angiotensinases. Solutions of this substrate were prepared in 0.1 M Tris–acetate buffer (pH 7.5).

Partially purified hog substrate. This was an extract of hog plasma, purchased from Miles Laboratories, Kankakee, Ill., which was found to contain no detectable renin, converting enzyme nor angiotensinases.

[*Asp*^{1}, *Ile*^{5}]*-tetradecapeptide.* This was of synthetic origin and was purchased from Schwarz BioResearch, Orangeburg, N.Y. It represents the N-terminal sequence of human and hog substrates[12]. Solutions of 0.5 mg/ml were prepared in 0.01 M

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acetic acid (pH 3.3) and their effective concentration was also verified by the enzymatic method.

pH curves

The incubation media consisted of 1 ml of Michaelis $^1/_7$ M barbital acetate buffer (pH 3–8.5) and, for reaction with hog substrate, of Soerensen $^1/_{15}$ M phosphate buffer (pH 5–8). Ionic strength (I) was constant at all pH values with the former buffer ($I = 0.16$) and varied slightly ($I = 0.006$ to 0.019) with the latter. A study of the effect of changes in ionic strength *per se* upon renin activity showed no significant effect between 0.01 and 0.02, while at 0.16 renin activity is about 1.7 times greater than at 0.01 (unpublished data). Thus within each pH curve, changes in ionic strength did not interfere. The concentrations of renin varied from $2.5 \cdot 10^{-3}$ to $5 \cdot 10^{-3}$ GU/ml and of the four substrates from 75 to 155 pmoles/ml, in order to obtain a formation of angiotensin in the same range. The substrates were incubated with renin in medium differing by 0.5 unit pH. After 30 or 60 min of incubation at 37 °C in siliconized tubes, aliquots of 20 to 200 µl were pipetted out and renin was inactivated in boiling water. Subsequently, cooling at 4 °C was found to be sufficient to inhibit renin activity, since no warming occured during subsequent procedures in the immunoassay.

Effect of pH on the substrate and on the enzyme

The purpose of these experiments was to determine the possible alterations of the different renin substrates by their acidification before incubation with renin. The technique of preliminary acidification is described in the legend of Table II. No change in ionic strength important enough to affect renin activity occurred in this procedure.

As a control, renin alone was submitted to the same acid treatment preliminary to incubation with the substrate at its optimum pH (only partially purified human substrate was used in this experiment). To study the possibility of a protective role of the enzyme preventing the acid denaturation of the substrate, partially purified human substrate was also acidified in the presence of renin.

Radioimmunoassay of angiotensin I

The aliquots pipetted out of the incubation media were made up to 1 ml with 0.1 M Tris–acetate buffer (pH 7.5) to which 10 µl ^{125}I-labelled angiotensin I and 10 µl diluted anti-angiotensin I antiserum were added. The technique of the ensuing radioimmunoassay has been previously reported[9,10].

RESULTS

pH curves (Table I, Fig. 1)

Four curves of renin activity corresponding to each substrate were obtained and presented a single peak at pH 4.5 for tetradecapeptide substrate (with a shoulder at pH 6), at pH 5.5 and 6 for non-purified and partially purified human substrate, respectively, and at pH 7.5 for hog substrate. It should be noted that renin activity with tetradecapeptide at physiological pH was negligible, but was still relatively high with human substrate (60% of maximum activity) and was at optimum with

TABLE I

pH CURVES OF HUMAN RENIN ACTIVITY WITH FOUR SUBSTRATES

The substrates were incubated with renin at 37 °C and at the different pH values indicated, at the following concentrations (pmoles/ml): 75 for tetradecapeptide substrate, 155 for plasma human substrate, 148 for partially purified human substrate and 125 for hog substrate. Renin concentration was $5 \cdot 10^{-3}$ GU/ml with the three latter substrates and $2.5 \cdot 10^{-3}$ with tetradecapeptide substrate. The results are expressed as the rate of angiotensin I formation (pmoles/ml per min).

pH of incubation	*Renin activity (pmoles/ml per min)*			
	Tetradecapeptide substrate	*Human substrate (plasma)*	*Human substrate (purified)*	*Hog substrate*
3	1.25	—	—	—
3.5	1.75	—	0.05	—
4	2.17	0.13	0.08	—
4.5	2.25	0.23	0.50	—
5	1.83	1.50	1.30	0.10
5.5	1.67	2.33	1.92	0.25
6	1.83	2.17	2.0	0.58
6.5	1.50	1.67	1.75	0.92
7	0.42	1.42	1.58	1.17
7.5	—	1.33	1.17	1.38
8	—	—	0.63	1.08
8.5	—	—	—	0.54

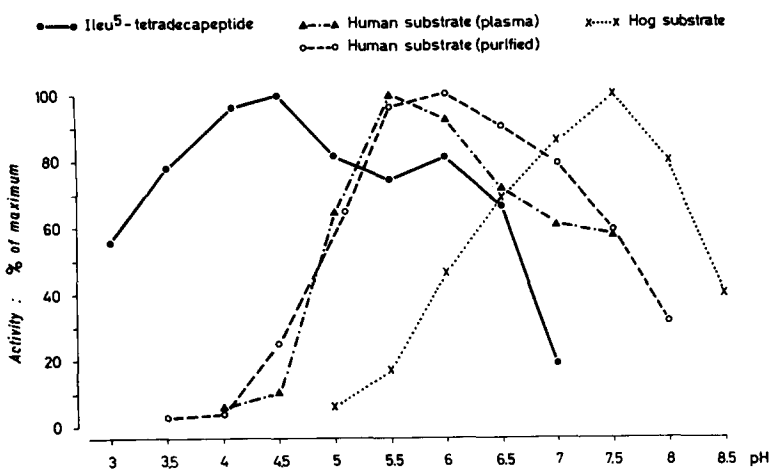

Fig. 1. pH curves of human renin activity with four substrates. The four substrates compared are: tetradecapeptide substrate (●—●), plasma human substrate (▲–·–▲), partially purified human substrate (○---○) and hog substrate (× · · · ×). The percentages of activity in ordinate correspond to absolute values given in Table I, where experimental conditions are reported.

hog substrate. On the contrary, at pH 4.5 where renin had its maximum activity with tetradecapeptide, human and hog substrates had lost most of their faculty to generate angiotensin I.

Effect of pH on the substrates and on the enzyme (Table II, Fig. 2)

The progressive acidification of the human substrate (either in plasma or partially purified) before incubation with renin led to a loss of activity proportional

TABLE II

EFFECT OF PRELIMINARY ACIDIFICATION OF THE FOUR SUBSTRATES AND OF THE ENZYME ON HUMAN RENIN ACTIVITY

The substrates have been acidified by addition of 0.1 M HCl and allowed to stand for 15 min at 4 °C at each of the pH values indicated. After centrifugation, the supernatant was readjusted to the optimum pH for each substrate (Table I) by adding 0.1 M NaOH. At that time, renin was added and the incubation at 37 °C started. The concentrations of the substrates and the enzyme were the same as in Table I. In the fourth column of results, partially purified human substrate (81 pmoles/ml) has been acidified with the same technique but in presence of renin ($5 \cdot 10^{-3}$ GU/ml). In the last column, human renin alone ($2 \cdot 10^{-3}$ GU/ml) was also submitted to acid treatment before incubation at optimum pH with partially purified human substrate (65 pmoles/ml).

pH of preliminary acidification	*Renin activity pmoles/ml/per min*					
	Substrates					*Enzyme*
	Tetradecapeptide	*Human (plasma)*	*Human (purified)*	*Human (purified) in presence of renin*	*Hog*	*Human renin*
2	2.25	0.15	0.08	0.27	—	0.10
2.5	2.38	—	—	—	0.75	—
3	2.38	0.45	0.22	0.42	—	0.50
3.5	2.13	—	—	—	1.0	—
4	2.13	1.10	1.58	0.93	—	0.52
4.5	2.25	—	—	—	0.94	—
5	—	1.65	2.38	1.50	—	0.47
5.5	—	1.95	—	—	1.12	—
6	—	—	2.51	1.58	—	0.50
6.5	—	—	—	—	1.16	—
7	—	—	—	—	—	—
7.5	—	—	—	—	1.15	—

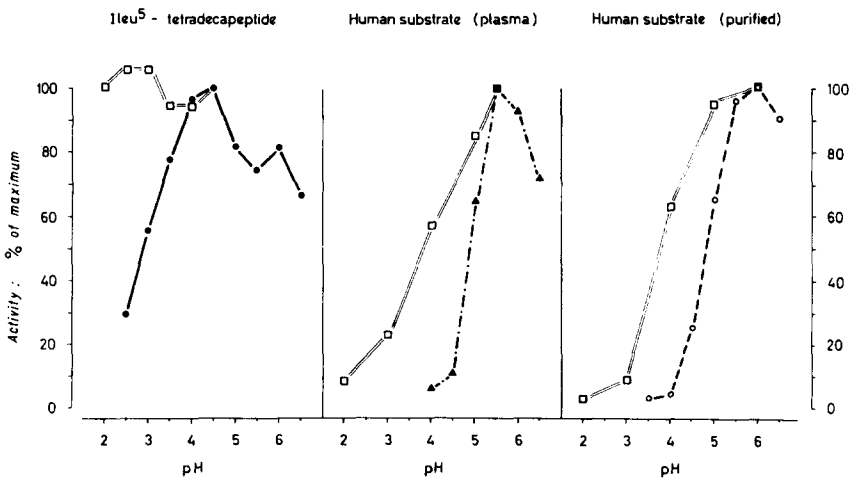

Fig. 2. Effect of preliminary acidification of substrates on human renin activity at optimum pH, compared to pH curves. The curves of acidification (□ = □) obtained by incubating at optimum pH the substrates previously acidified at various pH values, and the pH curves of the same substrates (symbols the same as in Fig. 1) are plotted using the same pH scale on the abscissa. The relative values of activity indicated on the ordinate correspond to absolute values given in Tables I and II, where the conditions of experiments are also mentioned.

to the pH decrease. This reduction of activity appeared below pH 5, amounted to 40% at pH 4 and about 80% at pH 3. The alteration of human substrate induced by acidification was irreversible; attempts to dissolve the light protein precipitate observed by readjusting the pH at optimum were unsuccessful, the results being identical to those in which the precipitate was discarded after centrifugation.

The tetradecapeptide was unaffected by the acidification in the zone studied where no loss of activity was noticed. If the denaturation curves were compared to the corresponding pH curves (Fig. 2), a relative parallelism was noted with human substrate, whereas the two slopes were clearly distinct with the tetradecapeptide.

In the case of human substrate, the denaturating effect of pH was less marked if the acidification was realized by an acid buffer than by progressive addition of a strong acid such as HCl.

When the renin alone was acidified before incubation at pH 6 with the partially purified human substrate, the activity remained stable from pH 6 to pH 3, but decreased at lower pH.

When the same substrate was submitted to the acid pretreatment in the presence of renin, a similar inactivation as reported above for the substrate alone was observed.

DISCUSSION

The present determinations of optimum pH values for human renin activity with different substrates agree with the results previously published. Helmer and Judson[1], Boucher *et al.*[2], and Pickens *et al.*[3] have obtained optimum pH values at about 5.5 with human plasma substrate. Skeggs *et al.*[4] and Rosenthal *et al.*[5] have obtained an optimum pH at 6 with purified human substrate. Gould *et al.*[6] and Dahlheim *et al.*[7] have described an optimum pH at 7.5 with hog substrate. Concerning the activity of human renin upon tetradecapeptide substrate, an optimum pH at 5.5, higher than the one of the present study, was determined by Waldhäusl *et al.*[13]. Skeggs *et al.*[4] isolated, from human kidneys and plasma, a "pseudo-renin" which was inactive upon plasma human substrate but active upon the homologous synthetic tetradecapeptide substrate with an optimum pH at 4.5 and a shoulder at pH 6, as in the pH curve obtained in this study. In the reported bioassays the tetradecapeptide possessed by itself a pressor activity[4], which had to be subtracted, whereas at the concentrations used the cross-reaction with angiotensin I was negligible in the present immunoassay. This method permitted a specific determination of angiotensin I, while with bioassays the formation of angiotensin has been measured without distinction between angiotensin I and II.

No explanation has ever been given for these differences of optimum pH with each renin substrate. As renin acts upon the four different substrates of this study by splitting the leucyl[10]–leucine[11] bond liberating the same decapeptide, it seemed evident that it was on the substrate itself that the pH exerted its influence. The distinction was particularly striking between the optimum pH of the complete human substrate and of its N-terminal sequence. The hypothesis of a direct effect of pH on the complete substrate was confirmed by the present experiments. The acidification preliminary to incubation produced an irreversible denaturation of the complete

sequence with a subsequent decrease of renin activity, whereas the tetradecapeptide was not altered by the same treatment. This difference of stability could explain the two optima observed with these two substrates. The greater stability of the hog substrate suggests a conformation or chemical structure different from that of human substrate.

On the other hand, after acidification the renin remained stable between pH 6 and pH 3. The enzyme showed no protective effect on the substrate since the same denaturation of human substrate was produced by acidification in the absence as well as in the presence of renin.

Such a denaturation of human substrate has been already described. When attempting to inhibit the angiotensinases by an acidification of human plasma, Helmer and Judson[1] noticed that a pretreatment at pH 3.9 before incubation at pH 5.5 produced a diminution of about 20% of renin activity. Skinner[8] demonstrated that human substrate was denatured below pH 4 at 32 °C and lost totally the faculty to generate angiotensin I below pH 3.5. However, these authors did not compare the pH curves of renin activity and of substrate denaturation. The present data give evidence for a relation between the two curves on the acid side, which allows one to explain the optimum pH of human renin activity with human substrate by a selective denaturating effect of pH on the substrate.

Since most assays of human plasma renin activity are achieved at pH 5.5 or 6, it is of practical importance to know the instability of human substrate at lower acid pH and consequently to pay attention to the mode of acidification of plasma, particularly with strong acids.

In conclusion, the present study demonstrates that various renin substrates present different behaviours under the influence of pH, although the enzyme, namely human renin, acts upon the same peptide bond, releasing the identical decapeptide angiotensin I. The discrepancy with regard to pH between the complete protein of human substrate and its N-terminal peptide sequence indicates that results obtained by using synthetic substrates with low molecular weight instead of natural substrates should be interpreted with caution.

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