Proteinase activity of human renin preparations: the International Reference Preparation (Renin Standard 68/356)

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(Received 7 May/28 November 1980; accepted 22 December 1980)

Summary
1. Several commonly used preparations of human renin, including the International Reference Preparation (Renin Standard 68/356), were examined for the presence of contaminating proteinase activity by using a 14C-glycinated bovine haemoglobin substrate assay.
2. All of the human renin preparations tested cleaved haemoglobin even in the presence of di-isopropylfluorophosphate and ethylenediaminetetra-acetate (EDTA). For a given amount of renin activity, varying amounts of proteinase activity were seen. The pH optimum also varied between preparations.
3. Small peptide inhibitors of human renin were not able to inhibit the proteinase activity. Furthermore a diazoacetyl reagent and pepstatin, both potent inhibitors of aspartic acid-active site proteinases, were only partially inhibitory.
4. These and other observations suggest that the proteinase activity of the human renin preparations is not due to renin itself, but to contaminating proteinases of different types. Since these enzymes may produce angiotensin I or peptides which may interfere in renin assays, crude preparations of renin which contain proteinase activity, including the International Reference Preparation, should be used with caution or replaced by proteinase-free human renin which can be easily prepared by use of suitable affinity chromatography.

Key words: affinity chromatography, proteinase, pseudorenin, renin.

Abbreviations: 14C-Hb, 14C-glycinated haemoglobin; EDTA, ethylenediaminetetra-acetic acid.

Introduction
Purification of human renin is complicated by contamination of the enzyme with other proteinases, the presence of which renders renin progressively unstable during its isolation. This problem has been overcome by the development of affinity chromatography which separates renin from the contaminating proteinases (Chou, Shaper & Gregerman, 1978) and has allowed the successful isolation of renin in a pure and stable state (Yokosawa, Inagami & Haas, 1978). However, the proteinase contamination problem has frequently been ignored, despite the fact that a variety of proteinases can cleave both natural and synthetic substrates in appropriate conditions and generate angiotensin I (Dorer, Lentz, Kahn, Levine & Skeggs, 1978; Hackenthal, Hackenthal & Hilgenfeldt, 1978). Thus misleading results may be produced in studies using crude preparations of renin. In the present paper we have studied the nature and magnitude of the proteinase contamination of several commonly used preparations of human renin, including the material available from the Medical Research Council as an International Standard (Bangham, Robertson, Robertson, Robinson & Tree, 1975).
Materials and methods

Materials

[1-14C]Glycine ethyl ester hydrochloride, specific radioactivity 9.8 mCi/mmol, was obtained from New England Nuclear. Bovine trypsin, porcine pepsin, glycine ethyl ester hydrochloride and three times recrystallized lysozyme were from Sigma Chemical Co., St Louis, MO, U.S.A. Bovine haemoglobin (two times recrystallized) and bovine albumin were from Pentex, Kankakee, IL, U.S.A. Diazoacetylglycine ethyl ester (diazoacetyl reagent) was the material prepared and described by McKown & Gregerman (1975). 14C-glycinated haemoglobin was prepared by the method of Williams & Lin (1971). Tetradecapeptide renin substrate (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) and tridecapeptide renin substrate (des-Asp-tetradecapeptide, Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) were from Schwarz/Mann, Orangeburg, NY, U.S.A.

Renin preparations

Two human renin preparations were kindly provided by Dr Erwin Haas. Lot 13 had a specific activity of 0.13 Goldblatt unit/mg of protein and lot 136 of 0.92 unit/mg. The International Reference Preparation of human renin was also studied (Bangham et al., 1975). In its method of preparation and specific activity, the International Reference Preparation is essentially identical with lot 136 (E. Haas, personal communication). Before use lot 13 was diluted in 1% (w/v) lysozyme and lot 136 in 2% (w/v) bovine albumin.

Quantification of proteinase activity

Each proteinase assay was performed in a final volume of 0.6 ml as described by Williams & Lin (1971). 14C-glycinated haemoglobin substrate (14C-Hb) was dissolved in the appropriate buffer so that 0.2 ml contained 1.5 mg or about 17,000 c.p.m. of 14C-Hb. The buffers used depended on the enzyme to be assayed: trypsin, Tris (0.5 mol/l, pH 8.1), containing CaCl2 (0.01 mol/l); pepsin, sodium citrate (0.1 mol/l, pH 3.0); renin, sodium phosphate (0.1 mol/l, pH 5.5 or as noted), containing ethylenediaminetetra-acetic acid (EDTA) (0.0025 mol/l) and di-isopropylfluorophosphate (0.0455 mol/l). Incubations were at 37°C, for 120 min with trypsin, 185 min with pepsin and 200 min with renin. Reactions were stopped by adding 0.5 ml of 10% (w/v) trichloroacetic acid along with 0.3 ml of 2% (w/v) bovine haemoglobin as a carrier. The solutions were centrifuged; 1 ml of the supernatant was counted in 10 ml of Aquasol (New England Nuclear Corporation, Boston, MA, U.S.A.) in a liquid-scintillation counter. Blanks (14C-Hb substrate incubated in buffer for the same time as the sample) averaged about 1% (150 c.p.m.) of the 14C-Hb substrate used. The degree of proteolysis, i.e. the precise number of peptide bonds cleaved, was not measured in this method. The solubilization of the 14C-Hb substrate is obviously related in some approximate manner to the magnitude of the peptide-bond cleavage. The assay results, when expressed as 'percentage proteolysis', recognize this limitation. The percentage proteolysis was obtained by measuring the counts per minute of the soluble fraction of the sample minus blank divided by the total counts per minute of untreated 14C-Hb minus blank.

Results

The proteolytic activities of human renin preparations, lots 13 and 136, as well as that of International Reference Preparation (Renin Standard 68/356) are shown in Fig. 1. The least pure preparation (lot 13) had the most proteolytic activity and, as expected, proteolytic activities of lot 136 and International Reference Preparation were similar and considerably less than that of lot 13. The proteolytic activities of trypsin and pepsin are presented in Table 1. 0.05 Goldblatt unit of lot 136 and International Reference Preparation had more proteolytic activity than 0.1 µg of pepsin and 0.1 unit of lot 13 had more activity than 10 µg of pepsin and less than 20 µg of trypsin. These experiments were repeated at least five times with different concentrations of renin and similar results were obtained.
Proteinase activity of renin preparations

The effect of pH on the proteolytic activities of renin preparations is shown in Fig. 2. The optimum pH for lot 13 was 3.2 and for lot 136 was 4.2. Similar results were obtained in a repeated experiment.

The inhibitory effects of pepstatin and diazoacyl reagent on proteolysis of renin preparations (lot 136) are shown in Fig. 3. As demonstrated even with $1 \times 10^{-5}$ mol of pepstatin/l only 55% inhibition and with $1 \times 10^{-1}$ mol of diazoacyl reagent/l only 48% inhibition of proteolysis were observed. A similar experiment was performed with lot 13 and maximum inhibition was 59% for pepsin and 66% for diazoacyl reagent. Repeated examinations for both preparations in the presence of added copper (which catalyses the inhibitory effect of diazoacyl reagent on renin action on its substrate) (McKown et al., 1974) produced even less inhibition (41 and 56% for lots 136 and 13 respectively), than in the presence of diazoacyl reagent alone.

The small peptide inhibitors of renin (e.g. Leu-Leu-Val-Tyr-OMe, Leu-Trp-Met-Arg-Phe-Ala, Leu-Leu-Leu) did not produce any inhibition of proteolysis even at the high concentrations used (3–6 mmol/l). The tetradecapeptide and tridecapeptide renin substrates also failed to inhibit proteolysis.

**Discussion**

When renin preparations containing equal amounts of renin activity were assayed for protease activity (Fig. 1), the lower specific-activity material (lot 13) showed more such activity than did the preparation of higher specific activity (lot 136). These results indicate that the less pure, lower specific-activity preparation (lot 13) was more heavily contaminated with proteinases (Fig. 1).

The differing degree of contamination was also emphasized by the pH–activity curves of the two preparations (Fig. 2); in addition this points to different types of contaminating proteinases.

Both preparations obviously contained large amounts of acid proteinases. When the International Reference Preparation of human renin was compared with lot 136, identical proteolytic activity was seen (Table 1). Because of the limited quantitative interpretation one can give the $^{14}$C-Hb assay, we examined the degree of cleavage in terms of known amounts of the reference proteinases trypsin and pepsin (Table 1). The degrees of proteolysis with the renin preparations were comparable with that observed with quantities of trypsin and pepsin shown. It is important to realize that this proteolytic assay

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**Table 1. Proteolysis of $^{14}$C-glycinated bovine haemoglobin by the renin preparation and reference proteinases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration</th>
<th>Proteolysis (%)</th>
</tr>
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<tbody>
<tr>
<td>International Reference</td>
<td>$5 \times 10^{-2}$ unit/ml</td>
<td>4.3</td>
</tr>
<tr>
<td>Preparation (Renin Standard 68/356)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>$20 \cdot 0 \mu g/ml$</td>
<td>69.2</td>
</tr>
<tr>
<td></td>
<td>$0 \cdot 1 \mu g/ml$</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>$1 \mu g/ml$</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>$10 \cdot 0 \mu g/ml$</td>
<td>47.4</td>
</tr>
</tbody>
</table>

The reaction conditions are as described in the text.
Therefore even cleavage of proteins by these preparations, which is undetectable in the proteolytic assay, could produce significant generation of angiotensin I in the renin assay. Similarly, interfering peptides could also be formed by proteolytic activity undetectable by our relatively insensitive assay.

In previous studies from this laboratory we found that several small peptides (e.g. Leu-Leu-Val-Tyr-OMe, Leu-Trp-Met-Arg-Phe-Ala, Leu-Leu-Leu) were inhibitors of human renin in a labelled polymeric substrate assay (Workman, McKown & Gregerman, 1974). To investigate whether the proteolytic activity of renin preparations on $^{14}C$-Hb was due to renin itself or to the contaminating proteinases, we tested the ability of these small peptides to inhibit proteolysis. No inhibition of proteolysis by these peptides was observed, even at the high concentrations used (3–6 mmol/l). Similarly, both the tetra- and tri-decapeptide renin substrates failed to inhibit proteolysis. Both peptides would have acted as alternative substrates for renin and would have produced apparent inhibition, if the proteolysis reaction were due to renin itself. We conclude, therefore, that the proteolysis is not due to renin, but to the contaminating proteinases. Others have also concluded that renin is not a general proteinase. Hackenthal et al. (1978) and Yokosawa et al. (1978) have reported that neither purified human nor rat renins exhibited acid proteinase activity with haemoglobin as substrate, although neither the concentrations of renin used nor the sensitivity of the protease assay was reported.

Renin, like other ‘acid’ or aspartic acid-active site proteinases (pepsin, cathepsin D etc.), is inhibited by diazoacetyl reagents (McKown & Gregerman, 1975) and the bacterial peptide, pepstatin (McKown, Workman & Gregerman, 1974). We have tested the inhibitory action of these two types of inhibitor on proteolysis of $^{14}C$-Hb by human renin preparations. Considerable inhibition was observed (Fig. 3), but neither pepstatin nor the diazoacetyl reagent produced complete inhibition of proteolysis. Since the activities of renin and cathepsin D are completely blocked by these two compounds, the proteolytic activity of human renin preparations on $^{14}C$-Hb must be due to contaminating proteinases. The nature of these enzymes, which are not inhibited by di-isopropylfluorophosphatate, EDTA, a diazoacetyl reagent or pepstatin, is not clear.

The extent of angiotensin I production by the action of different proteinases in human renin preparations acting on renin substrate cannot be predicted. However, the apparent action will depend on the experimental conditions and the sensitivity of the assay for angiotensin I. For example, Skeggs, Lentz, Kahn, Dorer & Levine (1969) have shown that ‘pseudorenin’ (almost certainly cathepsin D) does not react with angiotensinogen in the presence of serum, but will form angiotensin I when acting on purified angiotensinogen. Hackenthal et al. (1978) have demonstrated that cathepsin D and other closely related or identical proteinases (isorenin and pseudorenin) generate angiotensin I from angiotensinogen at a much slower rate than renin at pH above 6.

A number of investigators have used pepstatin affinity columns to remove acid proteinases during purification of human renin (Murakami, Inagami & Haas, 1977; Slater, Cohn, Dzau & Haber, 1978). Until more recently the products obtained were unstable presumably because of contaminating protease activity (Slater et al., 1978). Other preparations were highly purified, but only the short-term stability was described. Contamination by proteinases was said to be absent, but the assay used was relatively insensitive. This material was prepared by pepstatin affinity columns which would not be expected to remove all of the contaminating proteinases, as reported by others and as suggested by our present observations. However, since haemoglobin is a general substrate for proteolytic enzymes, a haemoglobin/Sepharose column was used in this laboratory to remove contaminating proteinases from human renin. Separation of ‘pseudorenin’ (cathepsin D-like activity) from human renin was readily accomplished (Chou et al., 1978). Use of such columns led to the preparation of pure proteinase-free human renin (Yokosawa et al., 1978).

Further work with renin should avoid use of renin preparations, including the International Reference Preparation, which contain proteinases. Although these preparations have been considered to be ‘angiotensinase-free’ under ordinary assay conditions, they are not proteinase-free. These contaminating proteinases are not entirely inhibited by ordinary proteinase inhibitors and may lead to the generation of angiotensin I or peptides which could be read as such in immunoassay, depending on the specificity of the antibody used. Such preparations may produce erroneous kinetic data (G. Pourmotabbed, H. J. Chou & R. I. Gregerman, unpublished data). The use of impure preparations of renin for the assay of renin substrate would appear to be
especially hazardous, since large amounts of the enzyme are used. Fortunately, the use of affinity columns should allow availability of proteinase-free human renin.

References


