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CHARACTERIZATION OF PROTEOLYTIC SYSTEMS IN HUMAN AND RAT URINE

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SUMMARY: Activities of proteolytic enzymes were detected in rat and human urine by using [^{125}I]iodo-insulin B chain as a substrate. The pH optimum of human urine activity was in the acidic range (pH 2.0) whereas the rat urine had two pH optima, one at the acidic range similar to human urine and another at pH 7.5. The activities were linear with time and amount of enzyme. Study with various proteinase inhibitors revealed that the acidic pH activities of human and rat urine were apparently of carboxyl endopeptidases since they were totally inhibited by pepstatin 10^{-8}M. The neutral pH proteolysis of rat urine was inhibited by chelating agents and therefore it was considered as a metalloendopeptidase activity. These findings show the difference between the content of urinary proteolytic enzymes in humans and in rats by using a sensitive and simple radioactive assay.

INTRODUCTION: Human urine is known to contain several proteolytic enzymes, mainly leucine aminopeptidase (1), alanine aminopeptidase (2), pepsinogen I group (3,4) and gamma glutamyl transferase (5). Determination of urinary proteinases has been used in the diagnosis of renal lesions (6,7).

The most common assays are based on colorimetric and fluorimetric methods with synthetic or natural substrates. However, in some cases the sensitivity of such methods is not sufficient in detecting low concentrations of enzymes in biological fluids. In this investigation, a radiolabeled protein, [^{125}I]iodo-insulin B chain, was used as a substrate. This peptide which is composed of 30 amino acids, is cleaved by various prote-

Abbreviations: EGTA; ethyleneglycol-bis-(ß-aminoethylether) N,N'-tetraacetic acid, EDTA; ethylenediaminetetraacetic acid, DTT; dithiothreitol, NEM; N-ethylmaleimide, PMSF; phenylmethanesulfonylfluoride, p-HMBA; p-hydroxymercuribenzoic acid, N.D.; not determined.

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inases with different specificities like cathepsins B, D, E, pepsin, chymotrypsin, trypsin, renin and papain (8). With [^{125}I]iodo-insulin B chain as a substrate it was possible to detect low levels of enzymes. In this report we have characterized the proteolytic activity of human and rat urine by determination of pH optimum, liniarity with time and amount of enzyme, and susceptibility to proteinase inhibitors.

MATERIALS AND METHODS

MATERIALS: Insulin, EDTA, PMSF, pepstatin, puromycin, antipain, leupeptin, chymostatin, iodoacetic acid, p-hydroxymercuribenzoic acid and $HgCl_2$ were obtained from Fluka (Buchs, Switzerland). EGTA, dithiothreitol, phosphoramidon and elastatinal were obtained from Sigma (St. Louis, USA). N-ethylmaleimide and O-phenanthroline were purchased from Merck (Darmstadt, FRG). Na^{125}I was obtaind from Amersham (Buckinghamshire, England).

METHODS

Urine collection: Human urine from healthy males and females was collected and stored at 4°C. The assay was carried out not later than 24 hours after sampling. Rats (Iva : SIV 50) of both sexes were put in metabolism cages for 17 hours, urine was collected in tubes containing 3 drops of thymol 10% in isopropanol for bacterial growth inhibition. This amount of thymol did not alter the proteolytic activity of the urine. The enzymatic activity was determined on the day of collection. Dilution of the urine with double distilled water was carried out before the experiment.

Proteolytic assay: [^{125}I]iodo-insulin B chain was prepared as previously described (9). The proteolytic assay (9) was carried out with the following modifications: Eppendorf tubes containing 60µl buffer, 20µl diluted urine and 20µl substrate (1nmole/20µl) were incubated for 20 minutes at 37°C. Control tubes contained 20µl of diluted boiled urine. To stop the reaction, the tubes were put in ice water, 100µl of casein 2.5% were added and the tubes were mixed. Then 200µl of trichloroacetic acid were added, the tubes were mixed again, and centrifuged for 3 minutes at 10000g. 300µl of the supernatant were counted in a Beckman Bio-gamma type II counter.

RESULTS

In order to characterize the proteolytic activities of human and rat urine, the first step was to determine the pH optima. As shown in Fig 1 the degradation of [^{125}I]iodo-insulin B chain by human urine was maximal at pH 2.0, whereas in the neutral and basic ranges, proteolysis was almost undetectable. In contrast, proteolysis of the iodinated peptide in rat urine had two pH optima, one at the neutral range (pH 7.5) and the second at the acidic range (Fig 1). There were no sex diferences in the

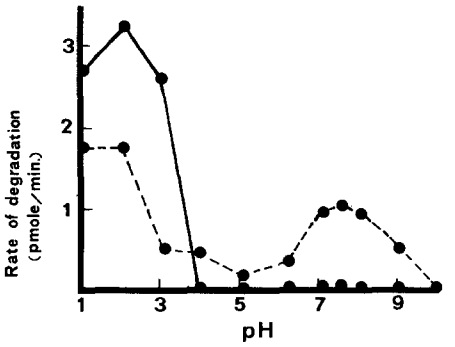

Fig. 1: pH dependence of the proteolytic activity of human and rat urine. Human (——) and rat (----) urine specimens (diluted 1/5 with water) were incubated with buffers as follows: pH 1-4 and 10 - glycine buffer, pH 5 - Na acetate buffer, pH 6-9 - tris buffer. The reaction was started by substrate addition and terminated after 20 minutes as described in Materials and Methods.

degrading activities (tested for six individuals of both sexes and species).

The degradation of [^{125}I]iodo-insulin B chain by human urine was linear up to at least 40 minutes, and there was a good correlation with urine concentration as well (Fig 2a). Similar results were obtained for rat urine at pH 7.5 (Fig 2b) and at pH 2.0 (Fig 2c). It should be noted that

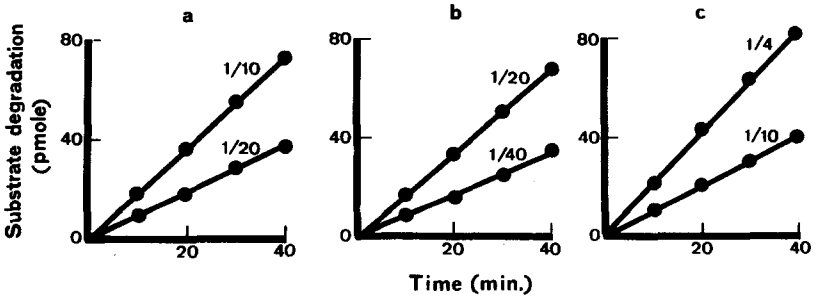

Fig. 2: Time and urine quantity dependence of proteolytic activities of rat and human urine.
Urine specimens were diluted with water as indicated and were incubated as follows: (a) male human urine with glycine-HCl buffer 0.2M pH 2.0, (b) female rat urine with tris-HCl buffer 0.2M pH 7.5, (c) female rat urine with glycine-HCl buffer 0.2M pH 2.0. The reaction volume was 1.0ml composed of 0.6ml buffer, 0.2ml diluted urine and starting the reaction with 0.2ml substrate (T=37°C). At the indicated times aliquots of 100µl were transferred into Eppendorf tubes containing 100µl casein 2.5% in water ice and the tubes were mixed. Then 200µl of trichloroacetic acid 25% were added, the tubes were mixed again, and centrifuged at 10000g for 3 minutes. After which 200µl of the supernatant were counted as described in Materials and Methods.

the proteolytic activity in highly concentrated urine was not linear with time and volume of urine. Therefore, appropriate dilution was used. For further characterization of the urinary proteolytic activities, the effect of proteinase inhibitors was studied. The results are summarized in Table 1. The neutral pH activity was inhibited significantly by

Table 1:Effect of proteinase inhibitors on the proteolytic activities of human and rat urine

inhibitor	concentation	human urine pH 2	rat urine pH 2	rat urine pH 7.5
		(% inhibition)		
EDTA	5mM	0	0	68
EGTA	5mM	6	0	88
O-phenanthroline	1mM	5	2	67
dithiothreitol	5mM	10	2	85
phosphoramidon	1mM	10	10	42
N-ethylmaleimide	5mM	5	0	3
iodoacetic acid	5mM	0	8	8
p-HMBA	2mM	47	30	17
$HgCl_2$	100µM	N.D.	30	43
	10µM	N.D.	21	2
PMSF	200µM	0	0	0
pepstatin	10nM	100	100	0
	200µM	100	100	0
puromycin	1mM	0	0	20
antipain	1mM	10	8	25
leupeptin	1mM	4	8	22
chymostatin	200µM	0	40	15
elastatinal	1mM	0	0	42
antipain + phosphoramidon	1mM + 1mM	N.D.	N.D.	65
EGTA + antipain	5mM + 1mM	N.D.	N.D.	91
EGTA + leupeptin	5mM + 1mM	N.D.	N.D.	90

Male human urine and female rat urine (diluted 1/3 with water) were incubated with the indicated buffers. Urine specimens were preincubated with the inhibitors for 20 minutes at 37°C. The reaction was carried out as described in Materials and Methods. O-phenanthroline and puromycin were dissolved in HCl 0.1N, iodoacetic acid and p-HMBA in NaOH 0.1N, PMSF, pepstatin and chymostatin were dissolved in dimethylsulfoxide. The other substances were dissolved in water. Control systems contained the solvents of the inhibitors. Results are the mean of at least two separate experiments.

chelating agents such as EDTA 5mM (68% inhibition), EGTA 5mM (88%), O-phenanthroline 1mM (67%) and dithiothreitol 5mM (85%), whereas these substances had no significant inhibitory effect on the acidic activity of both human and rat urines. Phosphoramidon, a neutral kidney brush border endopeptidase inhibitor (9), had a weak effect on the low pH proteolysis of rat and human urine, while the rat neutral activity was inhibited by 42% in the presence of this inhibitor. The thiol reagent p-hydroxy-mercuribenzoic acid partially inhibited the acidic pH activity of both human and rat urine (47% and 30% respectively), whereas the neutral pH activity was weakly affected by this substance. Another SH reagent, $HgCl_2$, was also a partial inhibitor for both activities of rat urine. Two additional thiol reagents, N-ethylmaleimide and iodoacetic acid did not change the proteolytic activities of both human and rat urine. Phenylmethanesulfonylfluoride, a serine protease inhibitor had no effect on the various proteolytic activities. however, the carboxyl endopeptidase inhibitor pepstatin (11), was a very potent blocker of the low pH activity in both rat and human urine. Pepstatin, in a concentration of 10^{-8}M, totally inhibited the proteolytic activity of urine at pH 2.0, whereas a high concentration such as $2X10^{-4}$M of the inhibitor was absolutely ineffective in rat urine at pH 7.5. Other proteinase inhibitors like puromycin, antipain and leupeptin had no effect at low pH, while the neutral activity of rat urine was weakly inhibited by these substances. Chymostatin, a chymotrypsin inhibitor, had no effect on human low pH activity, but that of rat urine was inhibited by 40%. The elastase inhibitor elastatinal partially inhibited the neutral pH activity of rat urine, whereas the acidic pH activity of rat and human urine was not affected. Combination of inhibitors such as antipain together with phosphoramidon which separately inhibited the neutral pH activity by 25% and 42% respectively, produced an additive effect so that in the presence of both the inhibition was 65%. In contrast, antipain or leupeptin had no additive effect when incubated together with EGTA. These results indicate

that more than one metalloendopeptidase are involved in the neutral pH activity of rat urine.

DISCUSSION

In this study we have characterized the proteolytic activities of human and rat urine by using [^{125}I]iodo-insulin B chain as a substrate. It was shown that the pH optimum of human urine proteolytic activity is in the acidic range (pH 2.0), whereas the rat urine activity had two pH optima, one at pH 2.0 like human urine and a second one at the neutral range (pH 7.5). These two activities of different pH values could be distinguished by their susceptibility to various proteinase inhibitors. The blocking effect of chelating agents on rat urine neutral pH proteolysis indicates that a metalloendopeptidase activity is present. On the other hand, in both human and rat urine, the acidic activity was totally blocked at a very low concentration by the carboxyl endopeptidase inhibitor pepstatin. In contrast, the neutral pH activity was resistant to this inhibitor even in high concentrations. These findings give additional evidence for the existence of two distinct proteolytic systems at the two different pH ranges.

Samloff et al (4) and Hanley et al (10) demonstrated the presence of pepsinogen in human urine. Since this enzyme belongs to the carboxyl endopeptidase group which is inhibited by pepstatin, it is likely that the proteolytic activity of human and rat urine at pH 2.0 is pepsinogen-like activity. In spite of the fact that both human and rat pepsinogen activities are fully inhibited by pepstatin, they differ in susceptibility to chymostatin. Human urine activity was resistant to this inhibitor, whereas the rat pepsinogen was inhibited by 40% in the presence of this blocker. This finding indicates that different kinds of pepsinogens exist in the urine of different species. Another interpretation for the inhibition by pepstatin is the involvement of cathepsin D and/or E. These proteinases are also inhibited by pepstatin, i.e. they belong to the carboxyl

endopeptidase group, and their pH optimum is in the acidic range. The possibility that renin, which is sensitive to pepstatin (11) , is responsible for the low pH activity can, however, not be excluded.

Determination of the proteolytic activities in urine is often used for the diagnosis and investigation of kidney damage caused by renal disease or by intoxication with a nephrotoxicant. However, comparisons of rat and human urinary proteinases revealed that there are significant differences between the two species. This should be kept in mind when the findings in rat are extrapolated in human.

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