

## HURAIN, A NEW PLANT PROTEASE FROM HURA CREPITANS

BY WERNER G. JAFFÉ

(From the Department of Chemistry, Instituto Químico-Biológico, Caracas-Los Rosales, Venezuela)

(Received for publication, March 15, 1943)

A considerable number of publications exist which describe plant proteases, but our knowledge of the chemistry of these enzymes and their mode of action is scant. Except for the ferments of the group of papainases, which have been relatively well investigated, there are few experimental data on other plant proteases, so that classification outside of this group is, as yet, impossible. In the present paper some experimental data obtained with a new plant protease are reported which may be helpful for the purpose of classification.

*Botanical Data*—The new ferment was isolated from the sap of the tree *Hura crepitans* (commonly known in Venezuela as *jabillo*) of the family of Euphorbiaceae. Proteases so far have been identified in three members of this family, *Croton tiglium*, *Ricinus communis*, and in the sap of *Euphorbia palustris* (1).

### EXPERIMENTAL

*Isolation*—When the bark and roots of *Hura crepitans* are cut, a brown, turbid, and caustic sap appears (which the natives use to remove bad teeth). This sap is slightly acid and has a pH of about 5 to 5.5. Sap isolated during the dry season yields 20 per cent of residue on drying; during the rainy season, about 17 per cent. It contains no caoutchouc. When centrifuged, the insoluble matter separates slowly and the supernatant liquid becomes clear; this, added to twice its volume of acetone, forms a white precipitate. The weight of the dried precipitate is 10.2 per cent that of the original sap. The precipitate was purified by re-solution and precipitation in the same manner. The resulting white powder is a protein and proteolytic enzyme herewith named "hurain." All the experiments were made with this crude preparation.

The insoluble matter, separated by centrifuging, was washed with water and recentrifuged until the wash water did not form a precipitate with acetone. The residue is a gray powder; its yield is 3 per cent of the crude sap. It may be further purified by solution in 1 N sodium hydroxide, filtration, and precipitation with acid. This purified residue shows the same proteolytic activity as hurain, and has been named "hurain *i*" (insoluble).

*Chemical Properties*—Hurain in aqueous solution is precipitated by satu-

ration with ammonium sulfate or sodium chloride; this precipitation is more complete if the solution is acid or alkaline. When alcohol is slowly added to an aqueous solution of hurain, precipitation begins when the alcohol concentration reaches about 30 per cent, and is completed when it reaches about 90 per cent. From acid solution, alcohol precipitates only part of the protein; the remaining fraction may be precipitated from the alcohol solution by neutralization. This fraction is also soluble in strong alkaline alcoholic solution.

Hurain gives positive Millon, xanthoproteic, and biuret reactions. Mineral acids, picric acid, mercuric chloride, and 0.1 M silver nitrate precipitate it. Sodium nitroprusside gives no color reaction with hurain. The iodine-azide reaction of Feigl for SH groups is positive, but this reaction is often positive for S—S groups too (2). When heated, hurain begins to coagulate at 90° and coagulates rapidly at 100°.

The viscosity of a hurain solution depends upon the pH. In slightly acid solution it has a low viscosity. When very slightly alkaline, it rapidly becomes so highly viscous that it is comparable to a pectin solution. In strongly alkaline solution the viscosity decreases again. In 5 M urea this change of viscosity is not observed. Similar observations have been made with gelatin solutions. In the latter case, too, urea inhibits the increase of viscosity which is observed normally when the solution is cooled (3). The hurain fraction which is soluble in acidified alcohol does not show this change of viscosity.

*Isoelectric Point*—An attempt was made to determine approximately the isoelectric point of hurain by means of a method (4) based on the fact that invert soaps precipitate only the anions of proteins (5). A concentrated solution of the ferment was mixed with different buffer solutions (McIlvaine's buffer) and a few drops of a 1:1000 invert soap solution (dimethyl-alkylbenzylammonium chloride) were added. The protein was precipitated between pH 4.25 and 4.6. The isoelectric point therefore probably lies between pH 4 and 5.

*Hurain i*—The water-insoluble hurain *i* is soluble in 1 N sodium hydroxide and in a borate buffer of pH 9. When acidified, it precipitates immediately from these solutions, but cannot be precipitated by boiling or by saturation with ammonium sulfate. It is also soluble in acid alcohol, and is precipitated by neutralization. It also dissolves in strong urea solution, and precipitates when the solution is diluted.

Hurain *i* is probably a secondary product. While the sap from the bark and roots of the tree *Hura crepitans* is turbid, the sap obtained from leaves and buds is clear and colorless but readily forms a white precipitate which has the same characteristics as hurain *i*. A similar product is obtained from hurain which has been kept for some time in strong alkaline solution.

*Proteolytic Properties*—To study the proteolytic properties, 10 per cent ferment solutions or the filtered sap (which also contained 10 per cent ferment) was used. To 0.25 ml. of these solutions, 0.25 ml. of activator or inhibitor solution, respectively, was added. The mixtures were kept at laboratory temperature (27°) for 24 hours. Then 9.5 ml. of the substrate solution were added and the whole mixture incubated at 37° for 6 hours. After this, formol titration was performed. As substrates, 2 per cent gelatin solution and 2 per cent peptone solution (Witte's peptone), which contained 10 per cent of 0.2 M dibasic sodium phosphate to adjust the pH to 8, were used.

### Results

Table I shows a comparison of the activities of hurain and ficin, both as natural saps which had been adjusted to contain 10 per cent of ferment.

TABLE I

*Comparison of Activities of Hurain, Hurain i, and Ficin*

The values give the quantity of 0.1 N NaOH employed in the formol titration.

Ferment	Substrate	0.1 N NaOH
		ml.
<i>Ficus</i> sap.....	Gelatin	2.15
“ “.....	Peptone	0.65
<i>Hura</i> “.....	Gelatin	1.80
“ “.....	Peptone	1.59
Hurain, 10% solution.....	Gelatin	1.66
“ 10% “.....	Peptone	1.52
“ <i>i</i> , 10% suspension.....	Gelatin	1.40
“ “ 10% “.....	Peptone	1.31

The *Ficus* sap used was a commercial product. It had the same activity as fresh papaya latex in 1:1 dilution. It appears that the gelatin-splitting activity of hurain is somewhat lower than that of the ficin, while the peptone-splitting activity is higher. Activation of the *Ficus* sap upon peptone could not be produced to any measurable degree.

Table II shows some results of investigations which were carried out in order to establish the activating and inhibiting action of a number of chemicals. It is evident that activators and inhibitors of papainases are without effect. Only four substances were found to influence markedly the activity of hurain, silver nitrate, mercuric chloride, iodine, and nitrous acid. Greenberg and Winnick (6) have pointed out that these same substances inhibit the activity of solanain, a proteolytic enzyme from *Solanum elaeagnifolium*. Nitrous acid was found by Philpot and Small (7) to reduce the activity of pepsin by 50 per cent. A similar reduction has been proved for solanain

and hurain. These three ferments give a yellow color reaction with the reagent.

Fig. 1 summarizes experiments to establish the optimum pH for gelatin-splitting of hurain. It was found to be approximately 8.

*Milk-Clotting Activity*—For the clotting of milk by hurain, the simple relation  $E \cdot t = K$  (where  $E$  is the quantity of ferment,  $t$  the time necessary to clot a constant quantity of milk at a given temperature, and  $K$  the constant of the process), which has been shown to express the clotting process

TABLE II

*Effect of Some Activation and Inhibition Substances on Hurain*

The values are expressed in per cent of the value obtained with the pure enzyme.

Substance		Gelatin digestion	Peptone digestion
	<i>M</i>		
Cysteine.....	0.1	98	101
Hydrocyanic acid.....	0.1	102	106
Hydrogen peroxide.....	0.1	100	98
Maleic acid.....	0.1	97	96
Phenylhydrazine.....	0.1	103	98
Iodine.....	0.1	7	3
Silver nitrate.....	0.02	8	18
Mercuric chloride.....	0.02	3	5
Nitrous acid.....	0.1	65	73

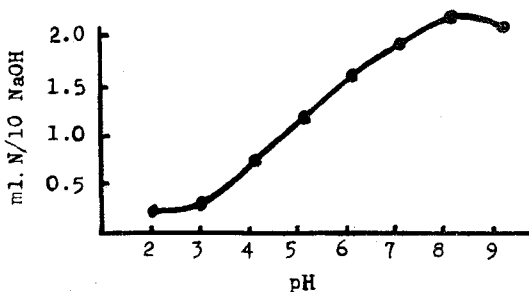


FIG. 1. Hydrolysis of gelatin mixtures, pH 2 to 9, by hurain

of chymotrypsin, applies, while the formula derived by Balls and Hoover (8) for papain does not.

The milk-clotting power of hurain is very low compared with its gelatin- and peptone-digesting activity. Fresh hurain has 5 milk-clotting units per gm. as defined by Balls and Hoover, if 5 ml. of milk are used at a temperature of 30°, and 30 units per gm. at 40°. For activated papain the corresponding values are 200 units per gm. at 30° and 300 units per gm. at 40°. Curve 1 in Fig. 2 expresses graphically the milk-clotting process of hurain and Curve 2 that of papain.

*Action on Intestinal Parasites*—Intestinal parasites are not attacked by

the proteolytic enzymes of the intestinal tract, owing to an antiferment which nullifies the action of these ferments and protects the parasites from their action. It has been demonstrated in recent years that papainases are not inhibited in their activity by these antiferments and therefore can digest living *Ascaris* and other intestinal parasites (9).

The action of hurain on *Ascaris* was determined by the following experiments. Three test-tubes were filled with 15 ml. of crude, unfiltered *Hura* sap and a fourth with crude *Ficus* sap. In the first tube were placed three living specimens of *Ascaris lumbricoides* from the human body; in the second, three dead worms of the same species; and in the third, three living earthworms. Three living *Ascaris* were put in the tube with the *Ficus* sap. All tubes were incubated at 37° for 18 hours. After this time the earthworms were dead and showed superficial ulcerations, while only one *Ascaris* had died and all appeared unattacked. The *Ascaris* in *Ficus* sap

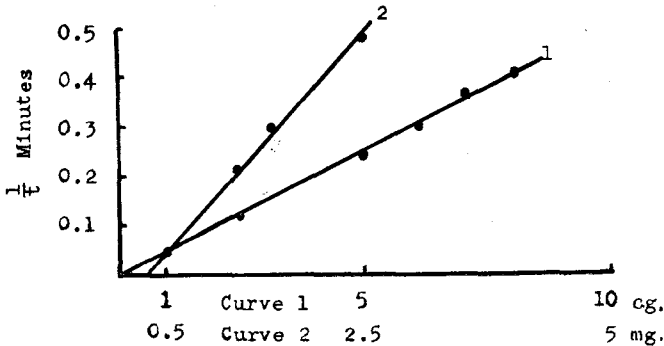


FIG. 2. Relationship between clotting time (reciprocal) and amount of enzyme. Curve 1, hurain; Curve 2, activated papain.

were strongly attacked and partly digested. The tubes were allowed to stand for 2 days more. By then, the earthworms were almost completely digested, forming a slimy mass, while all *Ascaris* appeared unattacked, but dead. The *Ascaris* in *Ficus* sap were as completely digested as the earthworms. It is evident from these experiments that hurain does not digest living or dead *Ascaris*, but digests earthworms. This difference certainly is due to the antiferments, characteristic of intestinal parasites like *Ascaris*.

*Toxicity*—Richet (10) studied the toxicity of the sap from *Hura crepitans*. He found that 0.1 ml. of the crude sap kills a rabbit within half a minute and that the toxic dose for dogs is 1 mg. of the crude protein derived from the sap per 100 kilos of body weight. This minimal dose killed the dogs within 3 days. He attributed this toxic effect to a toxalbumin which he named crepitin.

To check the toxicity, ten mice were given orally 0.5 ml. of the filtered *Hura* sap by means of a stomach tube. All animals survived the adminis-

tration for more than 8 days. To ten other mice was given 1 ml. each. These animals died within 24 hours. Stomach and intestines were filled with a slimy brown liquid. Anatomical lesions could not be detected. These results are not conclusive for oral toxicity, because death may be caused in mice by 1 ml. doses of solutions which are osmotically active or not neutral (11).

Of ten mice injected intraperitoneally with 0.1 ml. of the filtered sap, eight died within 24 hours, while of ten injected in the same way with 0.1 ml. of the sap diluted 1:1, only one animal died within 24 hours. There were no anatomical lesions visible. In the course of 1 week the two remaining animals of the first group and four more of the second group died. All had inflammation and necrosis in the peritoneum. All mice used in these experiments weighed between 16 and 18 gm.

Four rabbits were given intravenous injections of 1 ml. of the filtered *Hura* sap. All the animals survived the observation period of 4 days. Two rabbits were injected intraperitoneally with 5 ml. of the sap. These also survived 4 days, but all the animals of both groups died within 2 weeks. All the rabbits employed weighed between 1.5 and 1.7 kilos. The sap used in these experiments was collected in July. A sample obtained from the same tree in December was twice as toxic.

As toxalbumins derived from Euphorbiaceae have the property of agglutinating red blood cells, the *Hura* sap was checked with respect to this activity. It was found to agglutinate rat blood cells in a dilution of 1:100-000. Washed cells from human blood are agglutinated by the sap diluted 1:10 but not by a dilution of 1:100. No hemolytic action could be detected.

There exists a marked discrepancy between the toxicity of *Hura* sap found by Richet in Brazil and by the author in Venezuela. In both cases, however, a retarded action of doses insufficient to cause an acute toxic effect has been established. It has been shown that the toxic and the blood-agglutinating principles of ricin are not identical (12). If this is also true for crepitin, it is possible that the toxic factor is absent from the sap of *Hura* of Venezuela and that the retarded toxicity is due to a distinct factor. If the toxic effect is due to the toxalbumin crepitin of Richet, it must be present in a concentration lower than  $1^{-6}$  gm. per ml. of sap.

#### DISCUSSION

The most important characteristics of hurain may be summarized as follows: chemical behavior like that of an albumin; maximum of activity in alkaline range; no inhibition by merely oxidizing agents like hydrogen peroxide; positive Philpot and Small reaction; inability to digest intestinal parasites. These properties distinguish it clearly from the papainases. In the incomplete literature available to the author there is no description

of a plant protease with such properties. Nevertheless, it seems probable that among the great number of vegetable proteolytic enzymes, mentioned in the literature without detailed description, there are some which may possess the above characteristics. It is very probable that solanain recently studied by Greenberg and Winnick (6) may belong to the same group as hurain, because it shows a positive Philpot and Small reaction and has its maximum of activity at pH 8.5. It may be worth while to check the similarity of some other plant proteases to hurain. Perhaps a new group of these ferments may be established similar to the group of the papainases.

The papainases strongly resemble in their activity the catheptic ferments of animal origin. Vegetable ferments which are supposed to have analogous relations to pepsin have been found in *Drosera rotundifolia* (13). In the case of hurain a relation to trypsin may be suggested. All the characteristics summarized above are also true for trypsin. The reaction of Philpot and Small was checked for trypsin and found to be similar to that of hurain. Other characteristic properties of trypsin, especially the activation by enterokinase and by  $Mg^{++}$ , are as yet unchecked for hurain. As it is the purpose of this publication to describe some properties of the new enzyme which were easy and rapid to establish in order to find some eventual relationship to other plant ferments, no attempt to purify the crude product was made, and therefore these properties have not yet been checked for hurain.

## SUMMARY

A new proteolytic enzyme, named hurain, has been isolated from the tree *Hura crepitans*. Some of the properties which distinguish it clearly from the papainases are described. A possible relationship to trypsin is discussed.

## BIBLIOGRAPHY

1. Wehmer, C., *Die Pflanzenstoffe*, Jena, **2**, 674, 696 (1931).
2. Feigl, F., *Qualitative analysis by spot test*, Amsterdam, 184 (1937).
3. Signer, R., and Mosimann, H., *Helv. chim. acta*, **24**, 1058 (1941).
4. Jaffé, W. G., *J. Biol. Chem.*, **148**, 185 (1943).
5. Kuhn, R., and Bielig, H. J., *Ber. chem. Ges.*, **73**, 1080 (1940).
6. Greenberg, D. M., and Winnick, T., *J. Biol. Chem.*, **135**, 761 (1940).
7. Philpot, J. S. L., and Small, P. A., *Biochem. J.*, **32**, 542 (1938).
8. Balls, A. K., and Hoover, S. R., *J. Biol. Chem.*, **121**, 737 (1937).
9. Berger, J., and Asenjo, C. F., *Science*, **91**, 387 (1940).
10. Richet, C., *Compt. rend. Soc. biol.*, **66**, 663 (1903); *Chem. Abst.*, **4**, 595 (1910); *Ann. Inst. Pasteur*, **23**, 745 (1909); *Chem. Zentr.*, **1**, 1033 (1910).
11. Martin, G. J., Fisher, C. V., and Thompson, M. R., *Arch. Int. Med.*, **69**, 662 (1942).
12. Fuchs, L., and Falkensammer, H., *Sc. pharm.*, **10**, 103 (1939).
13. Holter, H., and Linderström-Lang, K., *Z. physiol. Chem.*, **214**, 223 (1933).