COMPARATIVE ACTION OF HURAIN AND TRYPSIN ON NATIVE AND DENATURED PROTEINS

BY

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(with 3 figs.)

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Hurain, the proteolytic enzyme of the sap of the jabillo tree *(Hura crepitans)*, has been reported to be activated by Fe^{++} and inhibited by Ni^{++}, Zn^{++}, Cd^{++}[1] and Cl^- ions[2]; its activity is also diminished in the presence of trypsin inhibitors from soy bean, black bean, *Ascaris lumbricoides*[3] and egg white[4].

A comparison between the activity of trypsin and hurain on synthetic substrates has been published[5]. In the present paper, some experiments on the action of these enzymes on native and urea denatured proteins are reported.

Material and methods

For the experiments described in this communication crude hurain powder obtained by lyophylization of the dialized centrifuged sap has been used. This material contains at least 6 fractions separable by paper electrophoresis, 4 of which have proteolytic activity.

Trypsin was a crystalline, salt free, commercial product purchased from Armour Pharmaceutical Company, Kankakee.

Bovine serum globulin (Cohn Fraction II and III) (Mann Research Lab., New York), casein (nach HAMMARSTEN) (Merck, Darmstadt) and edestin (Hoffmann-La Roche, Basel) have been used as substrates without further purification. Crude egg albumin (Merck, Darmstadt) solution was passed through a Sephadex G-25 column to eliminate an inhibitor fraction. Crystalline horse blood haemoglobin was prepared by HEIDELBERGER'S method[6]. A globulin fraction was prepared from kidney beans *(Phaseolus vulgaris)* by the method used by GOA and STRID[7] for the isolation of vicilin from peas.

1% solutions of these proteins were used in 1/15 M phosphate buffer of pH = 7.5 and in 6.6 M urea in the same buffer as substrates to compare hurain activity with that of trypsin. Proteolytic activity was determined by reading TCA (= trichloracetic acid) (6%) soluble products at 280 mμ.

TABLE I.

Comparison between the proteolytic activity of hurain and trypsin on native and urea denatured proteins.

No. Substrate	1. Trypsin activity		2. Hurain activity		3. Trypsin: hurain activities on denatured substrate	4. Trypsin: hurain activities on native substrate	5. Ratio of relative rates of hydrolysis. Trypsin: hurain on native substrate
	a. Units+/ mg	b. Relative rate of hydrolysis	a. Units+/ mg	b. Relative rate of hydrolysis			
In urea soln.	4	100	0.57	100	7:1		
1 Egg albumin in buffer soln.	0	0	0.44*	81*		0:0.44	0:81
In urea soln.	0	—	1.2	100	0:1		
2 Kidney bean globulin in buffer soln.	0	—	0.3	25		0:0.3	0:25
In urea soln.	25	100	0.6	100	41:1		
3 Bovine serum globulin in buffer soln.	0	0	0.1	17		0:0.1	0:17
In urea soln.	150	100	1.6	100	94:1		
4 Haemoglobin in buffer soln.	10*	6.6*	0.4	25		25:1	0.25:1
In urea soln.	27	100	1:1	100	27:1		
5 Bovine serum albumin in buffer soln.	3*	11*	0.13	12		25:1	1:1
In urea soln.	48	100	7.5	100	6:1		
6 Casein in buffer soln.	48	100	8.6	115		5.6:1	0.87:1
In urea soln.	55	100	0.9	100	62:1		
7 Edestin in buffer soln.	6	12	0.03	3		212:1	4:1

* Theoretical value (see text).

+) Definition see text.

The results were expressed in arbitrary proteolytic units, one unit being defined as the number of mg of enzyme protein that produce during — 30 minutes of incubation at pH = 7.5 products soluble in 6% TCA equivalent to an extinction of 0.100 at 280 mμ, measured in a 10 mm path of light in a Zeiss PMQII spectrophotometer.

Activities expressed in enzyme units were calculated from curves prepared for each enzyme-substrate system. In some cases, when maximum extinction obtained was lower than 0.100, the curve was extrapolated to this point and units calculated.

The result of the hydrolysis of a urea denatured substrate by an enzyme was considered 100% activity and that obtained from its action on the native protein was refered to this activity and expressed in percentage of activity of the denatured substrate. The value so calculated indicates a relative rate of hydrolysis of the native substrate compared with the denatured one.

Milk clotting activity was determined by the method of BALLS and HOOVER[5].

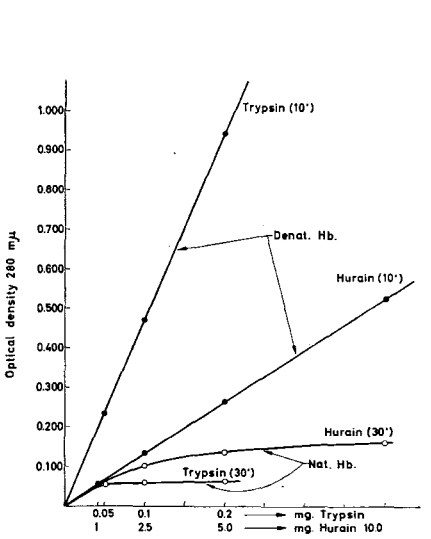
Results

The absolute activity of trypsin on urea-denatured proteins was always higher than that of hurain, probably due to the fact that the trypsin used was a pure crystalline product (Table I, Column 1a, 2a). The ratio of the absolute rates of hydrolysis of trypsin to hurain varied greatly, from 6:1 for casein digestion to 62:1 for edestin (Table I, Column 3).

Native egg albumin, bovine serum globulin and kidney bean globulin were not at all attacked by trypsin (Table I, No 1, 2, 3); native haemoglobin and bovine serum albumin were hydrolyzed only very slightly, the activity curve declining fast (Fig. 1), so that the activity values reported in Table I, Column 1 (No 4, 5) for these substrates are theoretical, calculated by extrapolation.

A marked difference between trypsin and hurain was observed with the kidney bean globulin fraction as substrate. No hydrolysis of the native or urea-denatured bean protein could be detected after 24 hours of incubation with trypsin. Hurain, on the other hand, hydrolyzed the native fraction to some extent, reaching an end point after 20 minutes of incubation. The denatured protein was attacked at a much higher rate by hurain, but was not at all hydrolyzed by trypsin (Fig. 2).

The relative rate of hydrolysis of 6 out of 7 native proteins used was higher for hurain than for trypsin, native edestin being the only exception (Table I, Column 1b and 2b). The relative reaction rate of trypsin and hurain on native substrates, as defined above, varied in a rather wide range, depending on the substrate, from



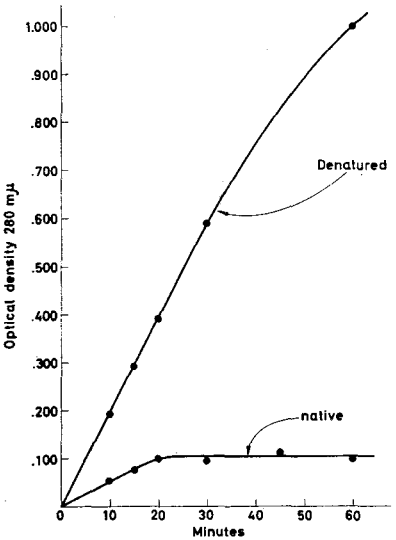


Fig. 1. Hydrolysis of native and urea denatured crystalline horse blood haemoglobin by trypsin and hurain.
Incubation periods are indicated on each curve. Test: 1 ml enzymes and 3 ml 1% substrate at pH = 7.5 were incubated. After 30 minutes 2 ml 20% TCA were added, the filtrate was read at 280 mμ in a 10 mm cuvette in a Zeiss PMQII spectrophotometer.

Fig. 2. Velocity of hydrolysis of native and urea denatured kidney bean globulin fraction by hurain.
Test: 5 mg hurain 3 ml 1% substrate solution of pH = 7.5 was used. At intervals, 3 ml aliquots were added to 2 ml 20% TCA. The filtrate was read at 280 mμ in a 10 mm cuvette in a Zeiss PMQII spectrophotometer.

1:2 for bovine serum albumin to 0:81 for egg albumin (Table I, Column 5).

Casein was hydrolyzed faster in buffer than in urea solution by hurain. This may indicate that hurain unlike trypsin-suffered a slight denaturation in urea solution.

The milk clotting activity of hurain and the influence of ions on it were determined (Fig. 3). Under similar conditions trypsin rather digested milk proteins than caused clotting.

Discussion

Hurain activity on urea-denatured proteins was quite different from that of trypsin (Table I, Column 3). In all cases but one, trypsin was more active than hurain, the first being a pure, crystalline product, but the ratio of their activity varied greatly, which

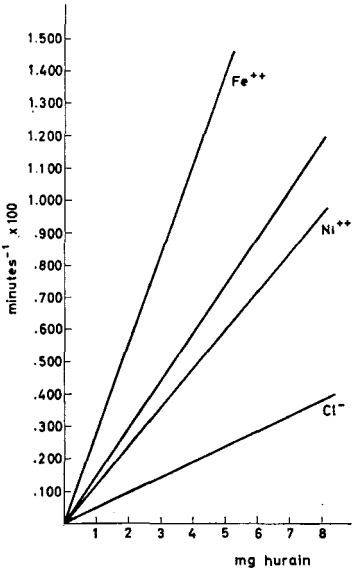


Fig. 3. Milk clotting activity of hurain. 0.5 ml of hurain solution, previously incubated with inhibitor or activators ions, were added to 2.5 ml 20% powder milk solution in acetate buffer of pH = 5.0. The onset of coagulation was observed directly in a water bath at 40° C, and results given in reciprocal values of clotting time. 10 μM Fe··, 10 μM Ni·· and 17 μM Cl′ were added respectively.

may indicate that the capacity of hurain to hydrolyze the substrates used was dissimilar to that of trypsin.

The difference between hurain and trypsin was most conspicuous when the substrate used was bean globulin as shown in Fig. 2. Whether these dissimilarities in enzyme action are related to those observed with synthetic substrates which proved that hurain had a broader amino acid specifity than trypsin has to be studied further. The fact that hurain is relatively more active on 6 out of 7 native proteins than trypsin is another point of dissimilarity. This property could make hurain a rather useful tool in the study of amino acid sequences of native proteins.

Milk clotting activity of hurain was detected by the BALLS and HOOVER method[8], and the effect of activating and inhibitor ions determined (Fig. 3), while trypsin quickly dissolved the clot by further hydrolysis of milk proteins.

BERKOWITZ-HUNDERT[9] classified proteases in rennet-like enzymes and common proteases not suited for curdle formation. Trypsin evidently belongs to the second group and hurain could probably be entered in the first.

As demonstrated above, hurain susceptibility to urea denaturation was greater than that of trypsin.

Hurain and trypsin are similar in respect to the lack of activation and stabilization by reducing agents. Their pH range of activity is also similar[10], and both are inhibited by trypsin inhibitors from animal and vegetal sources[3]. Based on these properties, JAFFÉ[10] suggested that hurain could be classified as a trypsin-like enzyme, and later IRVING and FONTAINE[11] included hurain, solanain and arachain in one group.

The activation of hurain by Fe^{++} and its inhibition by Ni^{++}, Zn^{++}, Cd^{++} and Cl', and the absence of an effect of Ca^{++} on its activity is probably the major difference between hurain and trypsin, as far as mechanism of enzyme action is concerned.

Different criteria have been used for the classification of proteinases. Groups of enzymes have been named after some particular representative, for example those activated by reducing agents are called papainases. The term pepsinases has been applied to any enzymatic material whose optimum proteolytic activity is at an acid pH and trypsinases to enzymes with their optimum activity at an alcaline pH. BERGMANN[13] proposed a classification based on the specifity towards certain synthetic substrates, enzymes having the same specifity as pepsin or trypsin respectively would be called pepsinases or trypsinases.

The similarity between hurain and trypsin in respect to pH optimum and some activators is counter-balanced by differences in the action of metal ions and in specifity towards synthetic and natural substrates. The possible classification of hurain must therefore await further studies.

Summary

The action of hurain, the proteolytic enzyme of the jabillo tree *(Hura crepitans)*, on native and urea-denatured proteins has been studied in comparison with trypsin.

The ratio of the absolute rates of hydrolysis of urea-denatured proteins by trypsin and hurain varied from 6:1 for casein digestion to 62:1 for edestin.

The hydrolysis value of an urea-denatured substrate by an enzyme was considered its 100% activity, and the readings obtained for digestion of the native protein were compared with this value giving a relative rate of hydrolysis of the native substrate. It was found that the relative rate of hydrolysis by hurain for 6 out of 7 native proteins was higher than for trypsin. It varied from 1:2 for bovine serum globulin to 0:81 for egg albumin.

Trypsin did not attack native or urea-denatured kidney bean globulin in 24 hours, while hurain hydrolyzed the native protein to

some extent, reaching an end point after 20 minutes incubation, and split the urea denatured globulin at a high rate.

The milk clotting activity of hurain was rather strong and was activated by Fe^{++} and inhibited by Ni^{++} and Cl^{-}. Under similar conditions trypsin did not produce visible milk clotting.

Hurain was found to be more susceptible to urea denaturation than trypsin, under similar conditions used.

The validity of the classification of hurain in the group of "trypsinases" is discussed.

Zusammenfassung

Die Wirkung von rohem Hurain, dem proteolytischen Enzym des "Jabillo" Baums *(Hura crepitans)* auf native und Harnstoff denaturierte Eiweisse wurde bestimmt und mit der von kristallisiertem Trypsin verglichen.

Die Bestimmung des Verhältnisses der Hurain-Wirkung auf natives und Harnstoff denaturiertes Eiweiss ergab in 6 von 7 Fällen höhere Werte als für Trypsin. Dieselben lagen zwischen 1:2 für Rinderserum-Globulin und 0:81 für Ei-Albumin.

Trypsin greift Bohnen-Globulin in 24 Std. weder im nativen noch im Harnstoff denaturierten Zustand an. Hurain hingegen hydrolisiert das native Eiweiss unvollständig und das denaturierte stark.

Hurain wurde von Harnstoff selbst geringfügig inaktiviert im Gegensatz zu Trypsin, das nicht beeinflusst wurde.

Die Milchgerinnungswirkung von Hurain war deutlich und wurde von Fe^{++} aktiviert, von Ni^{++} und Cl^{-} Ionen gehemmt. Trypsin ergab keine deutliche Milchkoagulation unter den angewandten Versuchsbedingungen.

Die Schwierigkeiten der Einordnung des Hurains in eine Gruppe von Trypsin ähnlichen Proteasen ist diskutiert.

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