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STUDIES ON A PROTEOLYTIC ENZYME FROM WITHANIA COAGULANS DUNAL

S.M. Amir, S. Masood Hasan and Sitwat Sultana

P.C.S.I.R. Laboratories, Karachi 39

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A proteolytic enzyme from fruits of *Withania coagulans* was isolated in a partially purified form and its properties studied. Evidence has been presented to suggest that the proteolytic activity is shown by the same milk-clotting enzyme, which had been obtained earlier from this source.

Withania coagulans grows commonly in the dry regions of Punjab, Sind and Baluchistan. Its fruit is employed as a remedy for dyspepsia and flatulent colic in the indigenous system of medicine.[1] The milk-coagulating property of the extract of pulp and seeds, contained in the capsular fruits, has been recognised for a long time.[2] A number of attempts have been made and described in the literature, to isolate the enzyme and to use it as a substitute for calf rennet in the preparation of cheese.[3,4,5]

Dastur *et al.*[6] who obtained the milk-clotting enzyme in aqueous extract of the pulp of the berries, found that extracts of seeds, leaves and stems had no clotting activity. They claimed that the cheese-making ability of their acetone precipitated enzyme, compared favourably with that of Hansen's liquid and powdered rennet.[5]

Atal and Sethi in 1961,[7] reported evidence for the presence of a proteolytic enzyme in the aqueous extract of the fruit, but the properties of this enzyme were not investigated any further.

During the present investigation, studies on a partially purified enzyme from the fruits of *Withania coagulans* were undertaken, and the effects of a range of inhibitors on the proteolytic and milk-clotting activities were compared.

Material and Methods

Substrate.—Casein and skimmed milk powder were used as substrates, for the determination of proteolytic and milk-clotting activities respectively.

Enzyme Solution.—All enzyme solutions used in the experiments were freshly prepared by extraction of the dried powder with the appropriate buffer. Occasionally the solutions were stored in deep freeze for short durations only.

Extraction of the Enzyme.—Dried berries (1 kg) of *Withania coagulans*, freed from husk, were ground and the pulp extracted with water (1.5 l) followed by a repeat extraction (750 ml). The combined extracts were adjusted to a pH of about 5.5, by slow addition of small portions of 1N sodium hydroxide. The mixture was kept overnight at 22° when a light brown precipitate was obtained, which was removed by centrifugation (15 min). To the clear supernatant solid sodium chloride was added to give a final salt concentration of 10%; the precipitate which contained some protein, besides other substances, was discarded.

The crude enzyme was precipitated from the supernatant by addition of sodium chloride (20%), and the mixture was kept at 22° for 24 hr. Thereafter, the residue was separated by centrifugation, taken in water (50 ml) and dialysed against water (1 × 50) for 40 hr at 22°. The enzyme was re-precipitated by addition of two-fold excess (v/v) of acetone and the mixture kept for 16 hr in the cold. Finally, the precipitate was centrifuged, washed with acetone and the residue dried *in vacuo* at room temperature.

Measurement of Proteolytic Activity.—The method used was a modification of that described by Kunitz[8] for the assay of trypsin. The UV absorption of the products of enzymic digestion was measured, following the precipitation of undigested casein by addition of trichloroacetic acid.

The dried enzyme (0.25 g) was always extracted with 0.1M phosphate buffer (pH 7) (25 ml), and the insoluble residue was discarded. The supernatant obtained was employed in the experiments described, while each individual set of experiments were carried out using the same enzyme extract.

The assay system for proteolytic activity consisted of 4 ml casein (1% soln) and 0.4 ml 0.1M phosphate buffer (pH 7) containing the enzyme. The blanks contained either the heat-denatured enzyme or the enzyme substrate mixture, to which trichloroacetic acid had been added before the start of incubation. The test and blank solutions were incubated at 37° for 20 min, when 3 ml 5%

trichloroacetic acid solution was added, and the mixture allowed to stand at room temperature for 0.5 hr. Thereafter, it was centrifuged, filtered and the absorbency of the clear filtrate measured at 280 mμ using 1 cm silica cells in a Unicamp spectrophotometre. In all subsequent experiments, unless otherwise stated, the proteolytic activity was measured by digestion of casein for 20 min at pH 7 and 37°.

Assay for Milk Clotting Activity.—12% solution of skimmed milk powder (5 ml) made 0.1 M with respect to calcium chloride was incubated with the enzyme solution (0.4 ml.) at 50°. The time required for the appearance of first clot was measured with a stop watch. The blank mixture contained the denatured enzyme.

Results

Properties of the Proteolytic Enzyme

(i) *Effect of Enzyme Concentration on Activity.*—Figure 1 shows the effect of enzyme concentration on proteolytic activity at pH 7 with casein as substrate. The reactivity was linear with respect to the volume of enzyme used, at least up to a value of 0.6 ml of the solution.

(ii) *Effect of Temperature on Activity.*—The enzyme reaction was carried out as described in standard conditions, except that the temperature of incubation was varied from 37° to 75°. The maximum proteolyti. activity was observed at 55°. The results are shown in Fig. 2.

(iii) *Effect of Time on Activity.*—The casein solution (1%; 12 ml) was mixed with the enzyme solution (5 ml) in 0.1M phosphate buffer pH 7, and the solution incubated at 37°. Aliquots (3 ml) were withdrawn after 10, 20, 30, 40, 50 min and immediately mixed with trichloroacetic acid solution (5%; 3 ml.). The absorbency of supernatant was determined at 280 mμ (Fig. 3) The initial increase in optical density was linear with respect to the period of incubation.

Effect of pH on the Activity.—Proteolytic activity was investigated at several pH values. Buffers employed for this purpose were 0.1M citrate, 0.1M phosphate and 0.1M borate. The enzyme showed greater activity at acidic pH and was completely inactive at pH 9 (Fig. 4).

Stability Against Heat Treatment.—Aliquots (0.4 ml) from enzyme solution were heated at temperatures of 40°, 60°, 80°, 100° for 5 min. Thereafter, they were immediately cooled in iced water and the residual proteolytic activity determined. The results are shown in Fig. 5. The enzyme was deactivated by heating, especially at elevated temperatures, and became complete inactive at 100°.

The reaction mixture containing the enzyme in 0.1M phosphate buffer (pH7) (0.5 ml), and metal solution (0.01M; 0.5 ml) were incubated for 1 hr at room temperature. The proteolytic and milk-clotting activities were determined as described earlier.

While Ag^{+} and Hg^{++} showed the maximum inhibition, similar effects were also observed to a varying degree, by the presence of Cu^{++}, Ca^{++}, Fe^{++}. However, K^{+}, Ba^{++} Pb^{++} and Na^{+} exerted little or no influence on the activity in the concentrations used. Of all the metals tested Ni^{++} alone showed a definite activating effect.

The reaction mixtures (1 ml), containing the enzyme (0.5 ml) in 0.1M phosphate buffer and 0.01M solution of the appropriate compound (0.5 ml), were incubated either for 1 hr or 0.5 hr at ambient temperature. The reaction of *p*-chloromercuribenzoate was carried out by treating the enzyme (2.5 ml) with the reagent in 0.1M phosphate buffer (pH 7). The effect of *p*-chloromercuribenzoate treatment was investigated at 0.005M and 0.01M concentrations. The proteolytic activities have been expressed in Table 2 as percent of the control system, containing no added chemical.

The sulfahydryl compounds, i.e. cysteine and glutathione, showed a marked activating effect both on proteolytic and milk-clotting activities, while the metal chelating agent, ethylenediaminetetraacetic acid (EDTA) as well as ascorbic acid, exerted only slight inhibitory effect.

p-Chloromercuribenzoate in 0.005M concentration, caused significant loss of enzymic activity. This effect, however, became more pronounced when the concentration was raised to 0.01M.

To the solution of the enzyme (2 ml) kept at 0°, saturated sodium acetate solution (2 ml) was added. Acetic anhydride (one drop) was then slowly added over a period of 1 hr. The reaction mixture was dialysed in the cold and the activities of dialysed solutions after dilution to a constant volume were determined.

In control experiment, enzyme was treated with sodium acetate alone. The results are shown in Table 3.

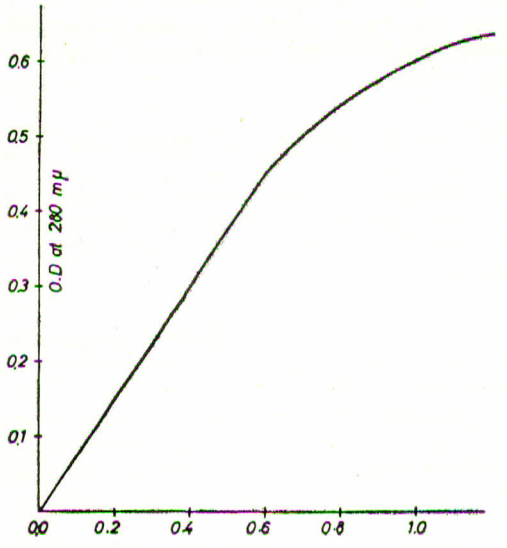

Fig. 1.—Effect of enzyme concentration on proteolytic activity.

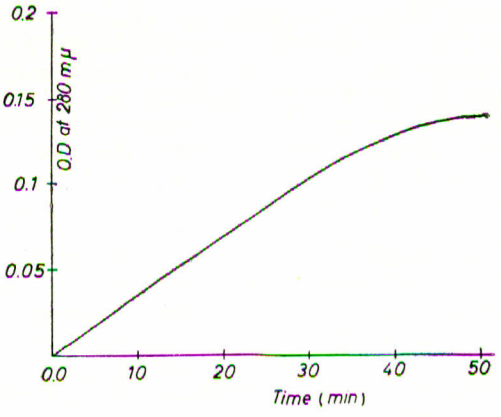

Fig. 3.—Effect of time on proteolytic activity.

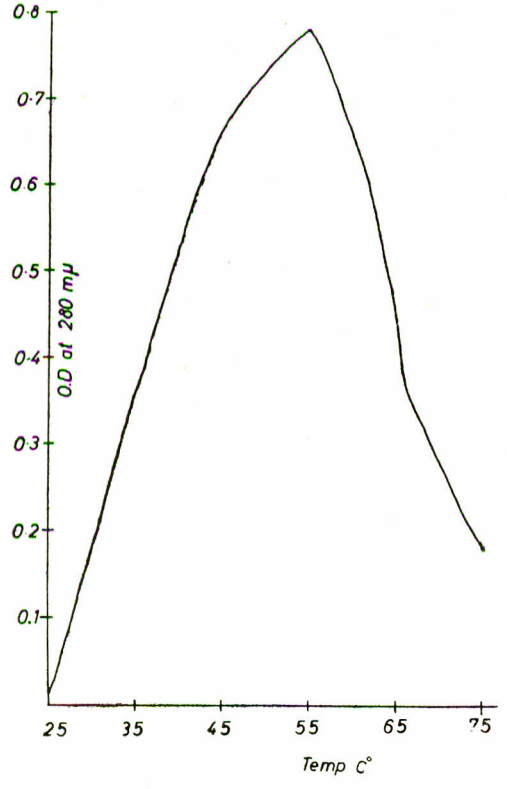

Fig. 2.—Effect of temperature on proteolytic activity.

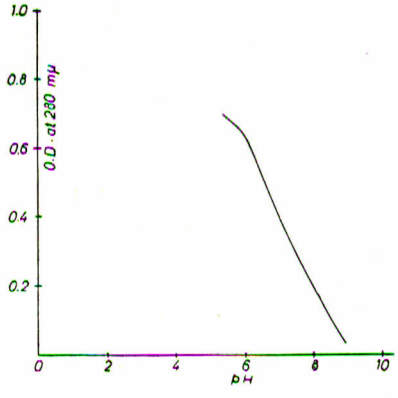

Fig. 4.—Effect of pH on proteolytic activity.

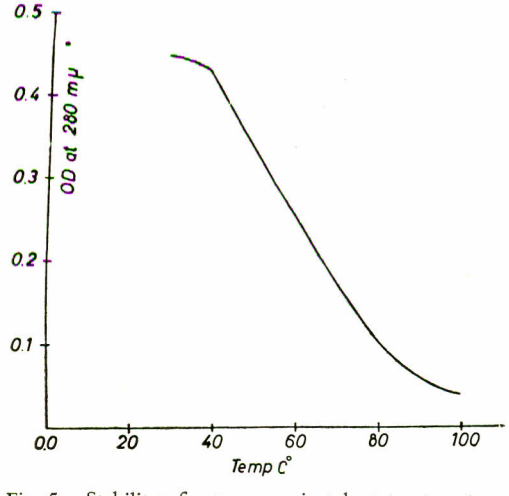

Fig. 5.—Stability of enzyme against heat treatment.

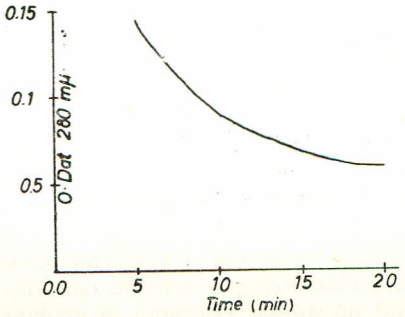

Fig. 6.—Stability of the enzyme against heat treatment at 60° for various lenghts of time.

TABLE 1.—EFFECT OF METALLIC IONS ON PROTEOLYTIC AND MILK-CLOTTING ACTIVITIES.

Salts (0.005M) added	Proteolytic activity expressed as % of control	Time taken for milk clotting (sec)
$NiSO_4$	124	13
$CuSO_4$	61	15
$BaCl_2$	104	13
KCl	104	13
$ZnCl_2$	91	15
$HgCl_2$	21	15
$Pb(NO_3)_2$	96	13
$CaCl_2$	90	13
NaCl	97	13
$FeSO_4$	78	17
$AgNO_3$	0	No clotting

TABLE 3.—EFFECT OF ACETYLATION AND DIALYSIS ON THE ACTIVITIES.

Treatment of the enzyme	Residual proteolytic activity as % of the original	Time required for milk-clotting (sec)
Acetylation	46	37
Untreated enzyme	100	42
Dialysis		
Against 5M urea	0	No clotting
Against 5M urea, followed by water	0	No clotting
Against water alone	69	50
Untreated enzyme	100	45

Acetylation[9] under condition stated above, resulted in 54% loss of proteolytic activity, but the milk-clotting ability of the enzyme was not effected appreciably.

Storage of the enzyme in 0.1M phosphates buffer pH. 7 for 2 weeks, whether in the frozen state or at room temperature, showed a loss of 22 and 49% of proteolytic activity respectively. Dialysis against urea solution also abolished the enzymic activity. Activity had been determined following the removal of the urea by dialysis. (Table 3)

Discussion

On account of the great lability encountered on storage and during purification procedure, it was not possible to obtain a purified specimen of the enzyme from *Withania coagulans*. All attempts to fractionate the crude enzyme at room temperature, by the use of sephadex G-200 and G-100 resins resulted in huge loss of activity. Considerable

TABLE 2.—EFFECT OF CERTAIN CHEMICALS ON PROTEOLYTIC AND MILK-CLOTTING ACTIVITIES.

	Activator/inhibitors	Concentration (M)	Proteolytic activity as % of control	Time needed for milk-clotting (sec)
(a)	*1-hr incubation*			
	Cysteine	0.005	123	36
	Glutathione	0.005	148	35
	No activator/inhibitor added		100	38
(b)	*p*-Chloromercuribenzoate	0.005	76	30
	p-Chloromercuribenzoate	0.01	53	69
	No activator/inhibitor added		100	22
(c)	*0.5-hr incubation*			
	EDTA	0.005	78	17
	Ascorbic acid	0.005	86	14
	No activator/inhibitor added	—	100	15

loss of activity was also noted on storage in the dried state for prolonged periods. Similarly, enzyme solutions in phosphate buffer pH 7 stored in the frozen state, deteriorated after a period of 2 weeks, while as much as 49% loss of activity was found to have taken place in the solution kept at room temperature.

It is now known that almost all proteolytic enzymes clot milk, although the degree of relative activities shown by a particular enzyme may be different.[10,11,12] Proteases such as trypsin, chymotrypsin, pepsin and papain, also possess milk clotting ability to a varying degree. The results obtained in the course of present work suggest that the milk clotting and proteolytic activities shown by the aqueous extract of the pulp of the fruits of *Withania coagulans* belong to the same enzyme. This is indicated by the fact that various chemical treatments and the large number of inhibitors or activator tested, influenced the two activities, in most cases, in a similar manner.

The optimum temperature for proteolysis was found to be 55° as compared to 45–65° reported for milk-clotting activity.[6] The present finding that greater proteolytic activity is shown as lower pH conditions, is also in conformity with the observation for milk-clotting enzyme recorded earlier.[6] Dastur *et al.*[6] have noted destruction of milk-clotting activity on heating the enzyme for 5 min at temperatures higher than 50°. A similar heat lability was also shown by the proteolytic enzyme in the course of our investigation.

The two activities showed the same degree of lability on storage and fractionation, while presence of cysteine and glutathione exerted a significant activating effect on both. Certain metal ions, particularly Ag^{+}, Hg^{++} and Cu^{++}, strongly inhibited the proteolytic activity while a similar but less pronounced effect could be observed on milk-clotting activity as well. Neither activity appeared to be influenced by the presence of Ba^{++}, K^{+}, Na^{+} ions. In this context it may be emphasized that whereas a high degree of reliability and accuracy can be obtained in proteolytic assays, the same was not possible in the milk-clotting assay.

The reaction with *p*-chloromercuribenzoate, which specifically modifies thiol groups of proteins, in 0.01M concentration, caused appreciable destruction of both the activities, indicating the presence of essential —SH groups in the molecule. Milk-clotting as well as the proteolytic activities were also inhibited, although to a lesser degree, when *p*-chloromercuribenzoate was used in 0.005M concentration. Complete loss of activity was observed by treatment with urea solution, when the enzyme was irreversibly denatured.

Acetylation of the enzyme modifies free amino groups present in the molecules, while hydroxyl groups of amino acids residues, such as serine and threonine, will also be acetylated to a limited degree.[13] Considerable loss of proteolytic activity on acetylation of the enzyme during our investigation, suggested the presence of susceptible amino acid residue in the active centre.

It is significant that Ca^{++} which has an activating effect on milk-clotting activity in concentration of 0.1M, had no similar effect on proteolysis. Also, addition of Ca^{++} ions in concentration of more than 0.1M had no further activating effect even on milk-clotting activity as is shown in Table 1. This is in agreement with the earlier finding, that Ca^{++} ion although activated the milk-clotting activity of an enzyme from soil, did not effect its proteolytic activity.[14]

It has been proposed[15] that primary action of all milk-clotting enzymes is to cleave κ-casein into para κ-casein and caseino-glycopeptide. The calcium ion by becoming bound to the former facilitates the process of milk-clotting, but has no effect on the initial degradation of the protein.

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