STUDIES ON THE MILK-CLOTTING ENZYME OF *STREBLUS ASPER*

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SUMMARY

1. Preparation and properties of the milk-clotting enzyme present in the leaves of *Streblus asper* are studied. Acetone was used as fractionating agent for the enzyme.

2. Studies on the enzyme before and after dialysis indicate that inactivation during dialysis and ultrafiltration are probably due to oxidation.

3. There is no linear relationship between the activity and enzyme concentration.

4. Boiled milk clots earlier than raw milk under the enzyme action.

Reports on the existence of milk-clotting enzymes in plant kingdom have appeared quite early in literature. The active principles of *Ficus carica* (Baginsky, 1882; Martin, 1885), *Withania coagulus* (Lea, 1883; Kothavalla, 1940; Yeshoda, 1941; Narain and Atma Singh, 1942) and *Galium varum* (Green, 1893) have been studied. Gerber (1907–13) in his extensive work on plant enzymes reported that the milk-clotting power of fig juice was known even to ancient Greeks (Gerber, 1907, 1913). Krishnamurti and Subrahmanyan made a study of the milk-clotting and protease components of *Ficus carica*. These authors also made a preliminary study of the enzyme present in *Streblus Asper*, a shrub which grows wild in the semi-arid regions of South India.

The present communication deals with the isolation and study of the properties of the active principle from *Streblus Asper*. The enzyme is present in all parts of the plant. In the following studies, the enzyme was extracted from the leaves.

METHODS

Preparation of the material.—Fresh leaves were cut into small bits and dried over anhydrous $CaCl_2$ in a vacuum desiccator for 24 hours. The dried material was powdered and passed through a 40-mesh sieve. The powder thus prepared, if stored in a tight-stoppered bottle in a dry place, will keep indefinitely without any loss in the enzyme activity.

Estimation of the enzyme.—20 gm. of leaf powder were steeped in 100 c.c. of distilled water and kept overnight in an ice-chest with the addition of toluene. The extract was filtered under suction. A dark brown coloured liquid was obtained. The extract could be kept in the refrigerator for two weeks without any loss in the activity.

Extraction with 5 per cent. sodium chloride and phosphate buffer at pH 7·0 was also tried and found to be of no special advantage over extraction with water.

Substrate.—The substrate used in these experiments was prepared using dried whole milk powder (Klim). 20 gm. of the milk powder were ground to a fine paste with a small amount of water and made up to 100 c.c. with water. After vigorous shaking it was filtered through cheese cloth. The milk was prepared fresh every day just before starting the experiment.

Determination of activity.—To 2 c.c. of milk in a dry tube (7·5 cm. × 1·4 cm.), 2 c.c. of M/5 sodium acetate-acetic acid buffer were added and mixed well. This was placed in a glass-walled thermostat maintaining 40° C. ± 1° C. for five minutes and 1 c.c. of the enzyme extract was added and mixed well. A stop-watch was started simultaneously. The tube was gently rotated and the time for the coagulation of the milk was noted. The average of three readings was taken in each case.

RESULTS AND DISCUSSION

Purification

1. *Dialysis.*—20 c.c. of the aqueous extract were dialysed against distilled water in a collodion bag for 24 hours at 5° C. After dialysis the extract was made up to 40 c.c. The dialysate was concentrated on a water-bath to 20 c.c. The activities of dialysable and non-dialysable fractions were tested according to the above method and the results are presented in Table I.

The results show that about 50 per cent. of the enzyme activity is lost on dialysis. The activity lost on dialysis cannot be restored by the addition of the dialysate.

TABLE I

Effect of Dialysis on the Milk-Clotting Activity

	Milk-clotting time in seconds
Aqueous extract	220
Dialysed extract	370
Dialysed extract + dialysate ..	370
Dialysate	No clotting

2. *Ultrafiltration.*—20 c.c. of the aqueous extract was filtered through a cellophane membrane No. 300 under a pressure of 60 kg. per cm.^{2}, and the residue on the membrane after complete filtration was removed by washing repeatedly with distilled water and made up to the original volume. The activities of the ultrafiltered fraction and ultrafiltrate were tested and the results are given in Table II.

TABLE II

Effect of Ultrafiltration on the Milk-Clotting Activity

	Milk-clotting time in seconds
Aqueous extract	220
Ultrafiltered enzyme	420
Ultrafiltered enzyme + ultrafiltrate	420
Ultrafiltrate	No clot up to 12 minutes

These results confirm the data obtained with dialysis. Hence neither the dialysis nor ultrafiltration could be employed as a method of purification of the milk-clotting enzyme.

3. *Purification by precipitation methods:* (*a*) *Precipitation with acetone.*—After preliminary fractionation studies with acetone, the following method of purification was adopted. To 20 c.c. of aqueous extract 4 c.c. of cold redistilled acetone were added and mixed well. It was allowed to stand for 5 minutes and centrifuged. The precipitate was taken in 20 c.c. of water and its activity was tested. This fraction was found to have no activity.

To the centrifugate 36 c.c. of acetone were added slowly with stirring and was allowed to stand for 5 minutes and centrifuged. The precipitate was taken in 20 c.c. of water and this fraction was found to contain the original activity.

(*b*) Precipitation with alcohol was also tried and it was found to have no additional advantage over acetone precipitation.

4. *Purification by adsorption methods.*—Various adsorbents, such as tricalcium phosphate, alumina C_r, kaolin, norite charcoal and amylose were tried. The enzyme could be adsorbed only on tricalcium phosphate.

The elutrients such as water, M/15 phosphate buffer of pH 7·0 and 1 per cent. ammonia were found to be inefficient in releasing the adsorbed enzyme from tricalcium phosphate.

Properties of the enzyme

The enzyme preparation obtained by fractionating the extract with acetone was used in the following studies.

Effect of pH.—The effect of pH on milk-clotting is shown in Table III. M/5 sodium acetate-acetic acid buffer was used for obtaining the pH in the acid range and M/5 phosphate buffer for pH range in the alkaline range. The reaction mixtures contained

2 c.c. of the substrate + 2 c.c. of each buffer + 1 c.c. of the enzyme.

TABLE III

Effect of pH on the Milk-Clotting Activity of the Enzyme

pH	Milk-clotting time in seconds
8·0, 7·5, 7·0	No clot upto 12 minutes
6·5	370
6·0	320
5·5	280
5·0	220
4·8	190

It is seen that the activity of the enzyme increased as the acidity of the reaction mixture increased. pH range below 4·8 was not studied since that would be too near the isoelectric point of the substrate (casein). In the rest of the studies reactions were carried out at pH 5·0.

Effect of enzyme concentration.—The relation between the enzyme concentration and activity is presented in Table IV. The reaction mixtures contained

2 c.c. of the substrate + 2 c.c. buffer of pH 5·0 + Varying amounts of the enzyme

total volume being maintained at 6·0 c.c.

TABLE IV

Effect of Enzyme Concentration on Milk-Clotting Activity

Volume of the enzyme	Milk-clotting time in seconds
0·4	342
0·8	230
1·0	198
1·5	156
2·0	145

There is no linear relationship between the activity and the enzyme concentration. The enzyme does not follow the law of Seglecke and Storch (1870). Briot (1907) and Bang (1900) reported that parachymosin does not follow the rule. Gerber (1907) attributed the discrepancy between the behaviour of parachymosin and rennin to the choice of unsuitable temperature range. Among plant enzymases papayotin and the enzyme of *Ficus carica* too do not obey the rule of Seglecke and Storch (Gerber, 1924). Bodanskey found that the chymase of *Solanin elængifolium* obeyed the above rule when the reaction was carried out at 37°, 47° and 55° C.

Influence of metal salts and other substances on the activity of the enzyme

The influence of certain metal salts, cysteine, thiourea and H_2S on the enzyme was studied. Suitable blanks were run to test the effect of the substances themselves on the substrate. The results are presented in Table V.

TABLE V

Substance added			Clotting time in seconds	Activation in per cent.	Inhibition in per cent.
Control	.. ..	..	240	..	..
$CuSO_4.5H_2O$	M/1000	..	525	..	118·7
$CuSO_4.7H_2O$	M/1000	..	210	12·5	..
	M/100	..	120	50·0	..
$MnSO_4.7H_2O$	M/1000	..	135	43·7	..
	M/100	..	65	73·0	..
$MgSO_4.7H_2O$	M/100	..	220	8·3	..
	M/50	..	185	23·0	..
$Na_2S_2O_3 \cdot 8H_2O$	M/1000	..	255	..	6·2
	M/100	..	355	..	48
$CaCl_2.6H_2O$	M/1000	..	240	..	..
	M/100	..	100	58·3	..
Iodine	M/1000	..	270	..	12·5
	M/100	..	No clot up to 12 minutes		
Cysteine	10 mgm.	..	105	56·2	..
	5 „	..	148	38·3	..
Creatinine	10 „	..	240	..	..
Thiourea	10 „	..	240	..	..
H_2S	10 „	..	172	28·3	..

The results presented in the above table show that M/1000 $CuSO_4$ lowers the enzyme activity by 65 per cent. Cobalt has activating effect at

a concentration of M/100. Manganese at a concentration of M/100 and M/1000 brings about activation. Iodine (M/100) completely inactivates the enzyme.

Effront (1917) reported that iodine has no action on animal rennet. Activation by cysteine and H_2S and inactivation by iodine suggest oxidisability of the enzyme.

It is clear from the table that M/1000 $CaCl_2$ has no effect, whereas M/100 $CaCl_2$ brings about activation. The part played by calcium has been differently reported by various workers. Hammerstein (1922) first put forward the view that rennin brought about coagulation only when certain concentration of calcium ions is present. Koestler (1925) also shared the same view. According to him a low calcium-ion concentration interfered with cheese making.

Effect of manganese and cysteine on the dialysed enzyme

On dialysis for 24 hours in cold the enzyme loses 50 per cent. of its activity. The effect of manganese and cysteine was studied to find out whether they can restore the activity lost on dialysis. The results are presented in Table VI.

The results show that the activity of the enzyme lost during dialysis can be restored by manganese ions and cysteine. From this it is clear that on dialysis the enzyme gets oxidized, and thus loses its activity. The fact that the dialysate or the ultrafiltrate when added does not restore the activity confirms that loss of the activity is not due to loss of any ions during dialysis.

TABLE VI

Effect of Cysteine and Manganese on the Dialysed Enzyme

Substance added	Milk-clotting time in seconds	Activation per cent.
Control (aqueous extract)	220	
„ (after dialysis)	370	
Dialysed enzyme + M/100 $MnSO_4$ ..	90	75·6
„ + M/200 „ ..	110	70·2
„ + M/1000 „ ..	300	18·9
„ + 10 mgm. Cysteine ..	190	48·7
„ + 5 „ „ ..	255	31

Action of milk-clotting enzyme on raw and boiled milk

The effect of the enzyme on raw and boiled milk was compared with its action on the synthetic substrate and the results are tabulated in Table VII.

TABLE VII

Substrate	Clotting time in seconds
Substrate (Klim)	210
Raw milk (cow)	240
Boiled milk (cow)	185

The results show that the clotting time is shortened when boiled (and cooled) milk is used as substrate. According to Briot (1908) the coagulation of fresh milk by the enzyme of *Ficus carica* is retarded due to the existence of anti-rennin which gets destroyed on heating. Gerber (1908) and Bang (1908) pointed out that the retardation is due to the presence of albumin and globulins. The inhibitory effect of these is removed when the milk is heated to a temperature of 65–80° C. According to them there is no anti-enzyme in raw milk. Animal rennin coagulated boiled milk less readily than raw milk. Bodanskey (1924) reported that the chymase isolated from *Solanium elængifolium* coagulated raw milk and some samples of boiled milk failed to be coagulated by the same enzyme. Thus it appears that the difference in the action of the enzyme on raw and boiled milk is not yet clear.

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