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The Activation of Papain and Related Plant Enzymes with Sodium Thiosulfate

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INTRODUCTION

A considerable number of substances have been studied as possible activators for papain. These studies led to the development of theories on the mode of action of these activators. As we found sodium thiosulfate to be a potent activator for papain and especially suitable for the study of the activation reaction, we shall present here some of our results. Moreover, thiosulfate may have some practical interest in connection with the industrial and medical use of papain.[1]

MATERIALS AND METHODS

The ferments used were mostly crude preparations. Papain and chymopapain were used both in the crude form and also after purifying according to the procedure of Balls and Lineweaver (2) or Jansen and Balls (3), respectively. However, our preparations were not crystallized. Ficin was obtained from samples of ficus sap by precipitation with acetone, and purified by redissolving and reprecipitating. In the same way, tabernamontanain (1) and the ferment from *calatropis gigantea* (4) were obtained. The latter will be referred to as "calatropain." Bromelin was prepared from pineapple juice according to the method of Balls, Thompson and Kies (5). The ferments were stored in well-stoppered glass bottles. For the experiments, freshly prepared 7.5% solutions were used. If necessary, these were diluted with reboiled distilled water.

The ferment activity was determined by the milk clotting method of Balls and Hoover (6) or the formol titration method. In the latter case, gelatin or peptone were used as substrates. Not all the experiments gave reproducible results. Only

[1] After the experimental work referred to in this paper had been finished, Dr. R. T. Major was so kind as to inform us that thiosulfates had already been described as activators for papain by Nies-Harteneck in German Patent No. 532,398. A preliminary note of our findings on the activation of papain by sodium thiosulfate has been included in a previous paper (1).

those results duplicated with at least two different enzyme preparations are mentioned in the following.

Results

The thiosulfate activation of the milk clotting action of 6 ferments of the papain group has been checked. The results are presented in Table I. These preparations were all partly inactivated by storage or treatment with H_2O_2. In every case, activation could be caused by

TABLE I

Activation of the Milk Clotting Activity of Different Ferments

Ferment	Activator	Clotting Time *minutes*
Papain	—	1.25
Papain	thiosulfate	0.57
Chymopapain	—	12.00
Chymopapain	thiosulfate	0.83
Ficin	—	2.75
Ficin	thiosulfate	0.42
Ficin	cysteine	0.46
Bromelin	—	15.00
Bromelin	thiosulfate	6.30
Tabernamontanain	—	2.33
Tabernamontanain	thiosulfate	0.42
Calatropain	—	60.
Calatropain	thiosulfate	1.7[illegible]
Calatropain	cysteine	1.68

0.1 cc. of a 7.5% ferment solution was mixed with 0.1 cc. of a 1/200 *M* activator solution and kept for 5 minutes at room temperature. Then 5 cc. of milk, prepared according to Balls and Hoover (6), at 40°C. were added to the mixture and the liquid kept at this temperature in a water bath until clotting occurred.

adding thiosulfate. The varying degrees of activation may possibly be a function of the ratio between reversible and irreversible inactivated enzyme present in each preparation and need not be considered as a characteristic of the different ferments.

The chymopapain, bromelin and calatropain preparations gave negative nitroprusside tests which remained negative after activation with thiosulfate.

Willstätter and Grassmann considered the activation of papain with cysteine to be a time-reaction (7). Scott and Sandstrom recently questioned Willstätter's finding (8). We present evidence in Table II

that activation of the milk clotting action of papain with cysteine, KCN, Na_2S and thiosulfate are time-reactions. Both the ferment solution and the activator solution were cooled to 5°C. They were then mixed, and milk at 40°C. added and the clotting time determined in the usual manner. In experiments 5–8 the ferment-activator solution was kept at 40°C. for 3 minutes before the milk was added. No full activation took place without incubation. The effect of incubation could be observed to a lesser degree with uncooled ferment-activator solutions. If a substantial excess over the minimum amount of activator necessary to produce full activation is employed, however, the effect is scarcely detectible.

TABLE II

Determination of the Influence of Incubation on the Activation of Ficin

No.	Activator	Clotting Time	Determination
		minutes	
1	—	4.25	immediately
2	cysteine	3.85	
3	KCN	3.33	
4	Na_2S	3.00	
5	thiosulfate	2.70	
6	cysteine	1.00	incubated 3 minutes
7	KCN	0.92	
8	Na_2S	1.18	
9	thiosulfate	0.83	

The cooled activator solution was added to the cooled ferment solution and the clotting time determined immediately, or after 3 minutes incubation at 40°C.

The quantitative relation between ferment and activating sodium thiosulfate was also studied. In Fig. 1 the result of such an experiment is presented. Curve 1 represents the relationship between the reciprocal clotting time and the amount of enzyme. It is virtually identical with the curves described by Balls and Hoover for papain (6). Curve 2 was obtained by plotting the reciprocal clotting time against the amount of thiosulfate used to activate 7.5 mg. of ficin. This curve shows that the activation is a reaction occurring in stoichiometric fashion. If the molecular weight of ficin is considered to be similar to that of papain—near 30,000—curve 2 indicates that equimolar amounts of thiosulfate activate equimolar amounts of ficin. The deviation of curve 2 from curve 1 is probably caused by the action of the residual inactive ficin. Balls and Hoover have shown that amounts of ferment

not sufficient to produce coagulation of a given amount of milk, will nevertheless effect later clotting of this same milk with a smaller amount of active enzyme than usual. This same effect probably causes curve 2 to deviate from curve 1.

There is a possibility that one molecule of ficin is activated by more than one molecule of thiosulfate, which could be concealed by impurities in the ferment preparation or by a higher molecular weight of ficin. But analogous results were obtained with a preparation of papain, which excludes this possibility.

The method of stoichiometric activation can be used to determine

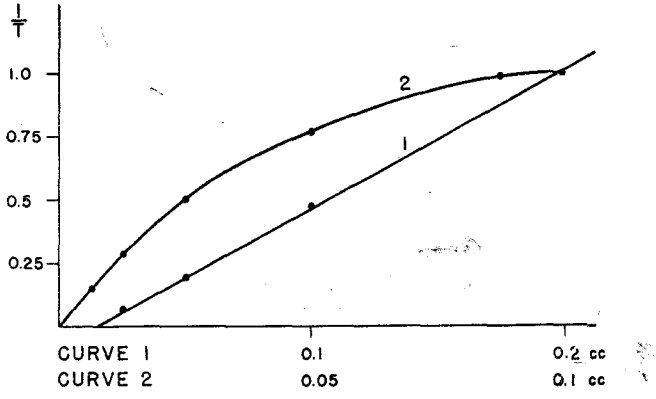

FIG. 1

Relationship between clotting time (reciprocal) and amount of activated ficin. Curve 1. 3.25% thiosulfate activated ficin solution. Curve 2. 1/400 *M* thiosulfate solution added to 0.1 cc. of 7.5% ficin solution.

the degree of purity in crude ferment preparations. In pure preparations, it can serve to detect the degree of reversible inactivation. In pure and reversible inactivated preparations, it may serve for crude determination of molecular weight in a manner similar to the method of quantitative inactivation with iodoacetic acid used by Balls and Lineweaver (2).

The speed of the reduction of S–S linkages to thiol groups depends upon the pH of the solution (9, 12). The reduction of S–S proteins is not believed to occur in an acid solution (10). Therefore, we checked whether activation would occur in a dilute acid. We mixed equal

volumes of ferment solution and $N/10$ HCl. The activator was then added and milk clotting time immediately determined with and without previous neutralization. The speed of the activation was not diminished to any detectible degree in the acid solution. The activities of the

TABLE III

Activation of Ficin in Acid Medium

Activator	$N/10$ HCl	$N/10$ NaOH	Clotting Time *minutes*
Na_2S	—	—	1.00
	0.1 cc.	—	0.58
	0.1 cc.	0.1 cc.	0.75
$Na_2S_2O_3$	—	—	0.92
	0.1 cc.	—	0.58
	0.1 cc.	0.1 cc.	0.88
—	0.1 cc.	—	4.10
—	—	—	4.30

The acid was added to the ferment solution before the activator solution. Neutralization was carried out immediately after the activator solution had been added, and clotting time determined.

acidified solutions were even greater than those of the solutions without added acid. Balls, Thompson and Kies made a similar observation (5). They found bromelin solution kept at an acid pH and later

TABLE IV

Activation of Gelatin and Peptone Splitting Action of Papain with Different Activators

Activator	Substrate	cc. $N/10$ NaOH
—	Gelatin	0.82
Thiosulfate		1.57
HCN		1.98
Cysteine		2.08
—	Peptone	0.57
Thiosulfate		1.18
HCN		1.30
Cysteine		1.10

0.5 cc. of a 5% solution of purified papain were mixed with 0.5 cc. of 0.01 M activator solution and kept for 1/2 hour at room temperature. Then 9 cc. of substrate solution (2% gelatin or peptone, respectively, adjusted to pH 6 with McIlvaine's buffer) were added. With 5 cc. of the final mixture formol titration was performed while the rest was incubated at 40°C. for 6 hours. After this time formol titration was again performed. The difference between the two values so obtained indicate the proteolytic activity and are given above.

neutralized was more active in coagulating milk than the same solution which had not been acidified previously.

Table IV summarizes the results of some experiments which prove that the gelatin and peptone splitting actions are also activated by sodium thiosulfate.

The plant proteases of the papain group usually occur in the plant juices together with a natural activator (11). This activator is believed

TABLE V

Influence of Different Activators on the Gelatin and Peptone Splitting Activity of Fresh Ficus Latex

Activator	Substrate	cc. $N/10$ NaOH
—	Gelatin	2.52
Thiosulfate		1.62
HCN		2.57
Cysteine		2.35
—	Peptone	0.65
Thiosulfate		1.28
HCN		0.53
Cysteine		0.59

Experimental procedure was the same as described in Table IV. Instead of ferment solution, crude ficus latex was used.

to be glutathione. The presence of such an activator is evident, for cysteine does not activate ficin in fresh ficus latex. If sodium thiosulfate is added to such a fresh ficus sap, the gelatin splitting action of the ficin is inhibited while the peptone splitting action is activated.

The milk clotting action of ficin in fresh ficus latex is equally inhibited by thiosulfate. In this case, it can be shown that the inhibition is a time reaction.

TABLE VI

Milk Clotting Action of Fresh Ficus Latex Diluted 1:10 with and without Added Activators

Activator	Clotting Time *minutes*	Determination
—	0.56	—
Thiosulfate	0.56	immediately
Cysteine	0.56	immediately
Thiosulfate	1.50	incubated 3 minutes
Cysteine	0.66	incubated 3 minutes

Discussion

Sodium thiosulfate activated all six studied papainase preparations in their gelatin splitting, peptone splitting, and milk clotting activity. A more detailed study of this activation gave some results which may be discussed briefly in respect to the mechanism of the papain activation.

Bersin and Steudel (12) have shown that the reduction of dithiol compounds with –SH compounds only attains equilibrium after several hours. Even when a high potential difference exists between ferment and activator one would, therefore, expect activation to be neither instantaneous nor quantitative. We have shown that the activation of ficin and papain with regard to their milk clotting activity was almost instantaneous. It has been proved that this is a time reaction, although with difficulty because of the high speed with which the reaction occurs.

The reductive splitting of S–S groups is described in the literature as strictly dependent upon the pH. Bersin and Steudel studied the reduction of dithioglycolic acid with cysteine and found a decrease of the reaction velocity with decreasing pH (12). Ryklan and Schmidt determined the oxidation potentials of a number of organic thiol compounds in a solution of their reduced and oxidized form and found them to increase with decreasing pH (9). Slotta and Fraenkel-Conrat inactivated snake poison with cysteine, a reaction which they believe due to the reductive breakdown of an S–S linkage in the protein molecule. This reaction did not occur in acid medium (10). The activation of papain and ficin, however, did take place in dilute acid. A decrease of the velocity of the activation was not detectible.

Thiosulfate does not reduce S–S bonds at a significant rate. When inactivated papain preparations, which did not give a positive nitroprusside test, were activated with thiosulfate the nitroprusside test remained negative. These facts can not be easily explained by the old theory of the activation of papain, which assumes the reduction of an S–S group.

Fruton and Bergmann discussed another hypothesis for papain activation (13). They believe that papain is a dualistic enzyme consisting of an apoenzyme and coenzyme. Activation, according to this theory, consists of the replacement of an oxidized active group by an activated one. The activation would involve the formation of a new

dissociable compound between ferment and activator. The authors based this theory on the observation that papain and cathepsin may be inactivated by dialysis or precipitation with butyl alcohol, and reactivated with cysteine, and also the HCN or H_2S activated enzyme preparations may be inactivated by removing these activators by evacuation.

Our results above cited can best be explained by Bergmann's theory. Likewise, our observation that thiosulfate activates the peptone splitting action of ficin in fresh ficus latex while, under the same conditions, its gelatin splitting and milk clotting actions are inhibited, can best be explained by the coenzyme theory. One must suppose that ficin, plus natural activator as an active group, is the more active in splitting gelatin and clotting milk, while the ficin-thiosulfate compound is the more strongly active in splitting peptone. Moreover, the observation that equimolar amounts of thiosulfate activate approximately equal amounts of papain, and the fact that activation takes place in an acid medium, best fit the coenzyme hypothesis of papain activation.

It must be mentioned, however, that during an experiment in which fresh ficus latex was dialyzed for 3 days against distilled water, a product was obtained which was still somewhat inhibited in its milk clotting action by thiosulfate. This may have been due to the crude method used because of lack of suitable dialysis equipment, although the dialyzed preparation gave no reaction with sodium nitroprusside.

Recently, the coenzyme theory of papain activation has been questioned. Winnick, Cone, and Greenberg did not observe a substantial loss of activity of ficin by dialysis when anaerobic conditions were secured (14). But, while Bergmann had used crystallized dipeptides as substrate to check the ferment activity, these authors worked with the substrate casein. Scott and Sandstrom studied the relationship existing between activator concentration and ferment activity, obtaining results which are opposed to ours (8). They found that maximum activation is obtained by certain excess amounts of activators. They concluded that papain activation may be a surface phenomenon. They used the substrate gelatin. From a comparison of these observations, it seems likely that results obtained with different substrates cannot be compared. The coenzyme theory would explain a substrate specifity of papain preparations activated with various activators as explained above. Such a specifity has been supposed by Fruton and Bergmann (13). But in view of the discrepancy in the results of the

different authors the possibility must be kept in mind that the activation mechanism of the various substrate specific activities of papain may be a distinct one. This may be due to the fact that papain is a mixture of different enzymes, or that it possesses various active centers which are activated in a different way.

Summary

Sodium thiosulfate activates the gelatine splitting, peptone splitting and milk clotting action of all 6 papainases checked.

The activation of the milk clotting action is a time reaction.

Approximately equimolar amounts of thiosulfate and ficin or papain react to form an activated ferment.

The activation of the milk clotting action will take place in an acid medium.

The gelatin splitting and milk clotting action of fresh ficus latex is inhibited by thiosulfate while its peptone splitting activity is stimulated.

The possibility of a different activation mechanism for the activity toward distinct substrates is discussed.

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