

## ON PROTEOLYTIC ENZYMES

XV. REGARDING THE GENERAL NATURE OF INTRACELLULAR  
PROTEOLYTIC ENZYMESBY MAX BERGMANN, JOSEPH S. FRUTON, AND  
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*Papain*

Papain has been shown to contain two partial enzymes (1) which will be designated in this paper as Papain I<sup>1</sup> and Papain II. Papain I is strongly inhibited by phenylhydrazine, while Papain II is not affected by this reagent. In the action of papain-HCN on gelatin at pH 5, both Papain I and Papain II participate in the proteolysis. If the same enzymatic hydrolysis is carried out in the presence of a sufficient quantity of phenylhydrazine, the splitting of gelatin is performed only by Papain II. The course of gelatin hydrolysis by papain-HCN in the presence or absence of phenylhydrazine is presented in Fig. 1.

It will be noted from Fig. 1 that Papain II is capable of carrying out an extensive degradation of gelatin even without the concurrent action of Papain I. Papain II hydrolyzes about one-half of all the peptide linkages.<sup>2</sup> The great extent of proteolysis by papain supports the view that the action of the proteinases is not confined to high molecular substrates.

The finding of the dual enzyme nature of papain suggested the hypothesis (1, 2) that the unactivated papain was a combination of the two partial enzymes and that the process of activation

<sup>1</sup> The term Papain I is employed to replace the previously proposed Papain Peptidase I.

<sup>2</sup> The splitting of all the peptide linkages would correspond to 12 mg. of NH<sub>2</sub>-N per cc.

involved a dissociation into two enzyme components. A series of experiments was performed to test this theory.

Papain preparations which have been freed as much as possible from the naturally occurring phytokinase by repeated treatment with hydrogen sulfide and alcohol (3) split gelatin quite rapidly to a moderate extent, and after a short interval no further change is

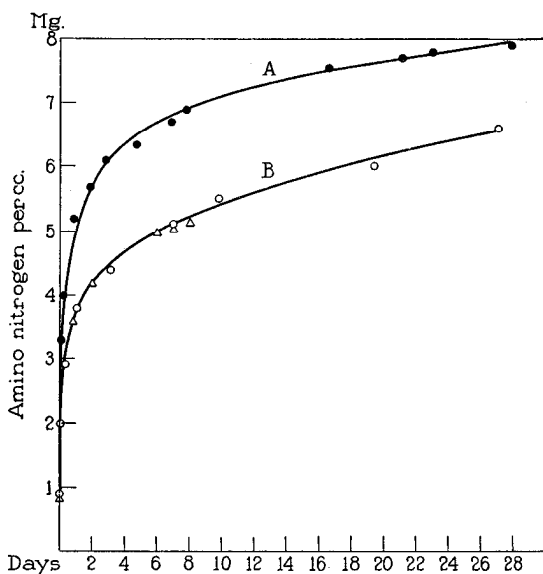


FIG. 1. Hydrolysis of gelatin by papain-HCN in the presence of phenylhydrazine. Curve A, 4 gm. of commercial papain + 120 gm. of Atlantic gelatin + 3 gm. of potassium cyanide + 250 cc. of citrate buffer of pH 5 + 750 cc. of water; Curve B,  $\circ$  as in Curve A + 10 gm. of phenylhydrazine,  $\Delta$  2.5 gm. of purified papain + 120 gm. of Atlantic gelatin + 3 gm. of potassium cyanide + 10 gm. of phenylhydrazine + 250 cc. of citrate buffer of pH 5.0 + 750 cc. of water. Temperature 40°.

observed (Fig. 2, Curve A). If to an enzyme preparation of this kind phenylhydrazine is added and is followed after some time by gelatin, there occurs a rapid and extensive hydrolysis of the protein (Fig. 2, Curve B). In a third parallel experiment the enzyme was first treated with phenylhydrazine to inactivate Papain I, and after several hours an amount of benzaldehyde was added which was equivalent to the phenylhydrazine. 2

hours afterwards gelatin was added and the resulting hydrolysis of the protein was very slight (Fig. 2, Curve C).

These experiments indicate that if Papain I is removed from the gelatin-papain system by means of phenylhydrazine, Papain II is automatically activated. The subsequent addition of benzaldehyde regenerates Papain I, but there results an inactive enzyme solution, not a mixture of active Papain I and active Papain II. The return of Papain I into the solution containing Papain II thus produces an extensive inactivation of the two partial en-

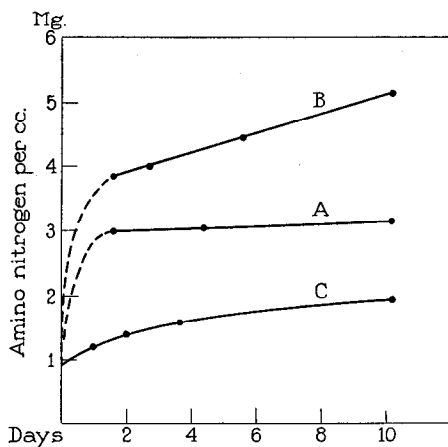
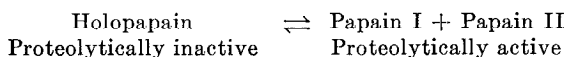


FIG. 2. Hydrolysis of gelatin by purified papain. Curve A, 0.4 gm. of purified papain + 12 gm. of Atlantic gelatin + 25 cc. of citrate buffer of pH 5.0 + 75 cc. of water; Curve B, as in Curve A + 1.5 gm. of phenylhydrazine; Curve C, as in Curve B + 1.5 gm. of benzaldehyde.

zymes. The two partial enzymes, when they exist in the same relative proportions as in the original papain preparation, inactivate each other so completely that only a slight initial splitting of gelatin can occur.

It was recently shown that with papain-HCN the results of such experiments are different (1). In this case there are active Papain I and active Papain II at the start. Addition of phenylhydrazine inactivates Papain I, and an equivalent amount of benzaldehyde regenerates this enzyme component; but because of the presence of HCN, Papain I and Papain II cannot inactivate each other.

In some of the recent literature (4) the activation of papain has been interpreted as a reduction process, and the reversible inactivation of the enzyme as an oxidation. In our opinion, this interpretation does not adequately explain the experiments with phenylhydrazine in which one of the partial enzymes is activated under the same conditions as the other partial enzyme is inactivated. The results of these experiments suggest strongly that the activation is to be considered as a dissociation in the following sense.



(Holopapain is the designation used for the compound of Papain I with Papain II.)

The question may arise whether, in addition to the dissociation, a reduction process plays some significant rôle in the activation. For Papain II it may be shown that after activation by phenylhydrazine the addition of HCN produces no additional activation. In Fig. 3 the action of papain-phenylhydrazine and papain-phenylhydrazine-HCN is compared. It will be noted that the presence of HCN had only a slight inhibitory effect.

It was reported (1) that Papain I, after inactivation by phenylhydrazine, cannot be reactivated by the addition of HCN. In Table I there are presented two experiments in which Papain I was first inactivated by means of different quantities of phenylhydrazine, then treated with HCN, and finally allowed to act upon carbobenzoxyisoglutamine at 40°. After 22 hours there was no noticeable splitting. However, the addition of cysteine or glutathione to Papain I-phenylhydrazine yields entirely different results. These sulfhydryl compounds are able to regenerate the activity of Papain I. It is possible that the reactivation by means of sulfhydryl compounds depends on a partial dissociation of the combination between Papain I and phenylhydrazine.

The activation of papain was first described by Mendel and Blood (5) who noted a more far reaching proteolysis of gelatin and other proteins by papain upon the addition of HCN or H<sub>2</sub>S. The action of these activators was believed by Willstätter and Grassmann (6) to consist in an extension of the specificity range of the enzyme. Following the discovery of a natural activator

which was present also in the commercial preparations of the enzyme, Grassmann and Dyckerhoff (7) expressed the view that

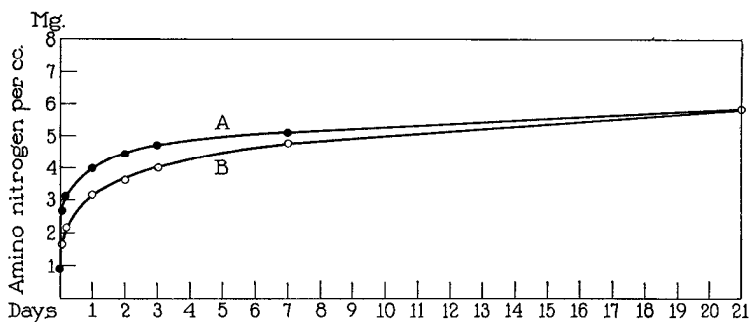


FIG. 3. Hydrolysis of gelatin by papain-phenylhydrazine in the presence of HCN. Curve A, 4 gm. of papain + 120 gm. of Atlantic gelatin + 10 gm. of phenylhydrazine + 250 cc. of citrate buffer of pH 5; Curve B, as in Curve A + 3 gm. of potassium cyanide.

TABLE I

*Hydrolysis of Carbobenzoxyisoglutamine by Papain I*

The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide linkage.

Reagent	Reagent per cc. solution	Hydrolysis		
		1 hr.	3 hrs.	22 hrs.
HCN.....	0.018	0.47		0.96
".....	0.018			
C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub> .....	0.02			0.01
HCN.....	0.018	0.39	0.55	0.76
C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub> .....	0.05			
Cysteine.....	0.005	0.25	0.36	0.71
".....	0.005			
C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub> .....	0.05	0.36	0.55	0.74
Glutathione.....	0.005			
".....	0.005	0.14	0.17	0.39
C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub> .....	0.05			

papain itself is proteolytically inactive but may be activated by different activators to perform different tasks. The limited ex-

tent of gelatin splitting by natural papain was interpreted by Maschmann and Helmert (8) as depending on the catalytic oxidation of the natural —SH activator by traces of copper in the gelatin. In view of these contradictory theories it seemed desirable to reinvestigate the effect of non-activated papain on gelatin.

In Fig. 4 there is reproduced the course of the digestion of a low ash gelatin preparation (Eastman Kodak) by a papain preparation which had been freed as completely as possible from the natural activators. The protein is split at the start but the proteolysis

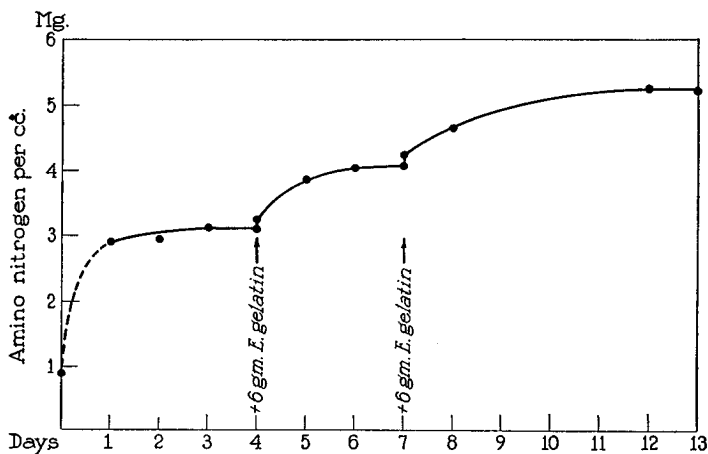


FIG. 4. Effect of addition of gelatin on papain hydrolysis. 0.25 gm. of purified papain + 12 gm. of Eastman gelatin + 25 cc. of citrate buffer of pH 5 + 75 cc. of water.

soon comes to a standstill. The addition of fresh gelatin produces a new brief digestion; a third addition of protein shows the same result. This experiment indicates that in the absence of activators papain attacks gelatin and its first degradation products but is inactive toward the smaller degradation products. A possible explanation for this behavior may be that gelatin and its first degradation products have so great an affinity to one or both of the partial enzymes of papain that they force a dissociation of the holopapain into the component enzymes. This high affinity would probably be lacking in the smaller degradation products; these cannot prevent the reformation of holopapain.

The foregoing experiments indicate the necessity of distinguishing between the substrates which are split by the enzyme without the assistance of activators and the substrates which require such activators. The enzyme which is inactive toward one substrate may be active toward another substrate. The expressions "active enzyme" and "inactive enzyme" therefore lead to an ambiguity in the case of papain.

*Synthetic Substrates for Liver Cathepsin and Bromelin*

In animal cells and organs there occur proteinases which may be activated by hydrocyanic acid and sulfhydryl compounds. These enzymes are designated cathepsins and are differentiated according to source as liver cathepsin, spleen cathepsin, etc. In order to compare the cathepsins of various normal and pathological tissues it is necessary to obtain simple substrates of known structure. It was possible to find that cathepsin from hog liver splits the following substrates: carbobenzyldiglycylglycine, carbobenzyloxy-L-leucylglycylglycine and its amide, carbobenzyloxyglycyl-L-glutamylglycineamide, and carbobenzyloxyglycyl-L-tyrosylglycineamide. The last named substrate is split at two peptide linkages. It is of interest, in regard to enzyme specificity, that carbobenzyloxyglycylglycylglycine is split more rapidly than the corresponding amide. Diglycyl-L-leucylglycine and triglycyl-L-leucylglycine are split by liver cathepsin with remarkable ease (Table II).

It will be noted from Table III that carbobenzyloxyglycylglycylglycine is split by cathepsin in the presence of cysteine to 100 per cent in 17 hours. The same substrate is scarcely attacked following the addition of phenylhydrazine. The originally active enzyme is thus inhibited by phenylhydrazine. In a third experiment, benzaldehyde was added following phenylhydrazine and then the action of the enzyme toward the substrate was regenerated. The enzyme which splits carbobenzyloxyglycylglycylglycine and is inhibited by phenylhydrazine may be designated Cathepsin I or, more precisely, Liver Cathepsin I. Cathepsin I behaves toward phenylhydrazine in the presence of activators in quite the same manner as does Papain I.

The cathepsin preparation employed effected no noticeable hydrolysis of albumin peptone within 5 hours. However, upon

addition of phenylhydrazine a strong hydrolysis resulted. A further experiment in which benzaldehyde had been added besides the phenylhydrazine showed only a slight hydrolysis. The com-

TABLE II

*Behavior of Synthetic Substrates toward Cathepsin and Bromelin*

The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide bond in the synthetic substrates.

Substrate	Cathepsin		Bromelin	
	Time	Hydrolysis	Time	Hydrolysis
	<i>hrs.</i>		<i>hrs.</i>	
Hippurylamide	21	0.01	23	-0.02
Benzoyl- <i>l</i> -isoglutamine	10	-0.02	23	0.01
	24	0.00		
Chloroacetyl- <i>l</i> -tyrosine	20	0.00		
	46	-0.01		
Carbobenzoxydiglycylglycine	10	0.28	23	0.01
	24	0.42		
Carbobenzoxy- <i>l</i> -leucylglycylglycine	20	0.84		
Carbobenzoxy- <i>l</i> -leucylglycylglycineamide	26	0.26		
	49	0.50		
Carbobenzoxyglycyl- <i>l</i> -glutamylglycineamide	20	0.23	20	0.78
	46	0.35	44	0.96
			68	1.05
Carbobenzoxyglycyl- <i>l</i> -isoglutamine			21	0.06
Carbobenzoxyglycyl- <i>l</i> -tyrosylglycineamide	26	0.50	29	1.45
	49	1.05	53	1.96
	72	1.37		
Benzoyl- <i>l</i> -leucyl- <i>l</i> -leucylglycine			21	0.04
Carbobenzoxydiglycyl- <i>l</i> -leucylglycine			21	0.01
Diglycyl- <i>l</i> -leucylglycine	21	0.97	21	-0.01
Triglycyl- <i>l</i> -leucylglycine	21	1.12	21	-0.01
Diglycyl- <i>l</i> -glutamylglycine	21	0.35	21	-0.02
Gelatin	4	0.58	2	0.87
	10	1.06		

ponent of liver cathepsin, which is not inhibited by phenylhydrazine and has properties similar to those of Papain II, may be designated Cathepsin II or Liver Cathepsin II.

On the basis of the same considerations as in the case of papain,

liver cathepsin may therefore be regarded as a dual enzyme, and its activation as a dissociation process. Both of the partial

TABLE III  
*Splitting by Cathepsin and Bromelin in Presence of Various Reagents*  
 The hydrolysis is measured in cc. of 0.01 N KOH per 0.2 cc. of solution.

Substrate	Reagent	Reagent per cc. solution	Cathepsin		Bromelin	
			5 hrs.	17 hrs.	2 hrs.	20 hrs.
Albumin peptone		<i>mM</i>	0.02		0.10	0.33
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.04	0.48		0.12	0.59
Gelatin	"	0.04	0.09			
	C <sub>6</sub> H <sub>5</sub> CHO	0.04				
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.04	0.35	0.95	0.42	1.11
	Cysteine	0.03	0.32	0.90	0.43	1.07
	"	0.03	0.42	0.95		
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.04				
	Cysteine	0.03				
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.04	0.54	1.48		
	C <sub>6</sub> H <sub>5</sub> CHO	0.04				
	HCN	0.05			0.77	1.71
"	0.05			0.35	0.83	
C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.04					
Carbobenzoxy-L-leucylglycylglycine	HCN	0.05				
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.04			0.57	1.54
	HCN	0.05				
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.04				
	C <sub>6</sub> H <sub>5</sub> CHO	0.04				
	Cysteine	0.03		1.00		
	"	0.03		0.05		
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.01				
	Cysteine	0.03				
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.01		0.91		
C <sub>6</sub> H <sub>5</sub> CHO	0.01					
Carbobenzoxyglycyl-L-glutamylglycine-amide	HCN	0.05				0.78
	"	0.05				
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.01				0.01
	HCN	0.05				
	C <sub>6</sub> H <sub>5</sub> NHNH <sub>2</sub>	0.01				0.52
	C <sub>6</sub> H <sub>5</sub> CHO	0.01				

enzymes participate in the splitting of gelatin in the presence of cysteine. Addition of phenylhydrazine inhibits Cathepsin I so that the initial splitting of gelatin is slowed down.

In 1930 Waldschmidt-Leitz *et al.* (9) reported the existence of a catheptic enzyme in hog liver, which, when activated, effected a slow splitting of chloroacetyltyrosine, benzoyl- and phthalylglycylglycine, carbethoxyglycylleucine, and perhaps leucylglycyltyrosine. This enzyme was considered to be a catheptic carboxypeptidase and its separation from cathepsin was described. Our crude enzyme preparation was completely inactive toward chloroacetyltyrosine under conditions in which carbobenzoxy-leucylglycylglycine was split to 84 per cent. Furthermore, our enzyme hydrolyzed carbobenzoxy-leucylglycylglycineamide and carbobenzoxyglycyltyrosylglycineamide which contain no carboxyl and therefore cannot be attacked by a carboxypeptidase. The enzyme belongs rather to the group of endopeptidases (proteinases).

The assumption of a special catheptic carboxypeptidase was required at a time when proteinases were not believed to be capable of attacking substrates of low molecular weight such as benzoylglycylglycine. Since this view has been found to be incorrect in numerous instances, there is insufficient basis at present to assume the existence of a catheptic carboxypeptidase.

It was found that the enzyme of the pineapple, bromelin, in the presence of hydrocyanic acid splits the following substrates: carbobenzoxyglycyl-*L*-glutamylglycineamide and carbobenzoxyglycyl-*L*-tyrosylglycineamide, the latter at two linkages (Table II). The splitting of carbobenzoxyglycylglutamylglycineamide is strongly inhibited by phenylhydrazine but is regenerated by benzaldehyde (Table III). The enzyme responsible for this behavior may be called Bromelin I. If in bromelin-HCN the Bromelin I is inhibited by phenylhydrazine, there still remains another active enzyme which effects an appreciable splitting of gelatin. This enzyme may be called Bromelin II.

The bromelin preparation employed showed an appreciable splitting of albumin peptone even without the addition of activators. However, an addition of phenylhydrazine produced a greater rate of splitting. Thus, the relationship between the two component enzymes of bromelin is essentially similar to the case of papain or cathepsin.

The question has been raised in the literature regarding the number of different papainases. It has been suggested that all

the plant papainases are identical to the enzyme from *Carica papaya* and, in particular, that papain and bromelin are identical enzymes (10). It was possible to test this view by means of the synthetic substrates. A comparison of the data presented in Table I for Cathepsin I and Bromelin I with previously published results for Papain I shows great specificity differences among these three enzymes. For example, activated Cathepsin I does not attack benzoylisoglutamine in contrast to Papain I. Furthermore, activated bromelin is inactive toward a series of peptides and peptide derivatives which are split by papain.

#### EXPERIMENTAL

The preparation of the synthetic substrates employed in this paper has been described in previous publications of this series.

The commercial papain preparation (1) was purified by precipitation with alcohol according to Grassmann (3). It was found that 2.5 gm. of the purified papain were equivalent in enzymic potency to 4 gm. of the commercial preparation. Cathepsin was prepared by desiccating pig liver with alcohol, extracting the dry material with 87 per cent glycerol, and filtering (9). The bromelin was prepared from pineapple juice by ammonium sulfate precipitation (10).

Cathepsin was activated by cysteine just before use for hydrolysis; bromelin was incubated with HCN for 2 hours at 40° for activation. In the phenylhydrazine experiments the reagent was left in contact with the enzyme solution for 2 hours before testing. A similar interval followed the addition of benzaldehyde; the benzaldehyde phenylhydrazone was centrifuged off, and the clear supernatant liquid was employed.

The concentration of the synthetic substrates was in all cases 0.05 mm per cc.; the concentration of peptone and gelatin was 47 mg. per cc. and 40 mg. per cc. respectively, except where otherwise stated. The amounts of the enzymes used in the experiments with synthetic substrates were as follows: cathepsin, 0.5 cc. of the glycerol extract per cc. of test solution; bromelin, 4 mg. of the dry preparation per cc.; papain, 2.25 mg. of the dry preparation per cc. Cathepsin experiments were maintained at pH 4.0 to 4.2; bromelin and papain experiments, at pH 5.0. The temperature was 40° in all cases.

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