ON PROTEOLYTIC ENZYMES

IX. THE INACTIVATION OF PAPAIN WITH IODINE

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When papain is treated with iodine, the activity of the enzyme against gelatin is known to be lost (1, 2). It is possible, however, to restore at least a part of the activity by treatment with HCN. It was recently found that papain splits not only proteins, but also a series of simple synthetic substrates such as hippurylamide, benzoylisoglutamine, and acylated peptides (3, 4). It was shown furthermore that the activity of papain against hippurylamide could be suppressed by iodine exactly as is its activity against gelatin. When the iodine-oxidized papain was subsequently treated with HCN, it became active against gelatin, but no activity against hippurylamide could be found. The experiments reported in this paper have been performed in order to ascertain whether the difference in the behavior of reactivated papain against various substrates is also to be found when the inactivation of papain is performed with the minimum amount of iodine.

At present, a papain preparation is at our disposal, which, before HCN activation, has an appreciably greater effect on gelatin than our earlier preparations had. Therefore, we investigated the effect of the new preparation on synthetic substrates. Benzoylisoglutamine was used in addition to hippurylamide for comparative purposes, since it is split faster by papain.

In Table I three papain solutions, which were obtained from two different solid papain preparations, are compared. Papains I and II were prepared from the same solid papain. The extraction of the solid material was accomplished with greater thorough-

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ness in the case of Papain II. Papain III is the preparation used in the experiments reported in Paper VIII of this series (5). After activation with HCN, its activity against gelatin is comparable with that of the present preparation; however, before HCN activation it is much less active than the present preparation.

TABLE I

Activation of Natural Papain

Enzyme	Substrate	Hydrolysis in ml. of 0.01 N KOH					
		1 hr.	2 hrs.	4 hrs.	6 hrs.	18 hrs.	24 hrs.
Natural Papain I	Gelatin	0.46					1.03
HCN-Papain I	"	0.73					1.49
Natural Papain I	Hippurylamide				0.04		0.09
HCN-Papain I	"				0.54		0.96
Natural Papain I	Benzoylisoglutamine		0.25				0.65
HCN-Papain I	"		0.62				0.93
Natural Papain II	Gelatin	0.63	0.84		1.06	1.20	1.19
HCN-Papain II	"	0.89	1.07		1.29	1.59	1.61
Natural Papain II	Hippurylamide			0.03			0.10
HCN-Papain II	"			0.55			0.95
Natural Papain III	Gelatin		0.36				0.85
HCN-Papain III	"		0.90				1.68
Natural Papain III	Hippurylamide		0.03				0.05
HCN-Papain III	"		0.21				1.06

In every experiment the solution contained for each ml. of volume 0.05 mM of synthetic substrate or 40 mg. of gelatin, 0.1 ml. of 0.02 N disodium citrate buffer (pH 5.0), and 0.2 ml. of enzyme solution. Benzoylisoglutamine was dissolved in the equivalent amount of 0.5 N sodium acetate. The temperature was 40°. The digestion was measured in ml. of 0.01 N KOH per 0.2 ml. of solution, according to the method of Grassmann and Heyde (6). 1 ml. represents 100 per cent splitting for all synthetic substrates.

Both preparations were obtained in Ceylon from the milky sap of *Carica papaya* by careful vacuum evaporation, and were kept in storage at a temperature of approximately 5°.

As may be seen from Table I, those preparations of natural papain which, even before HCN activation, have a very strong

effect on gelatin manifest also a distinct splitting of hippurylamide. But the extent of this splitting is, indeed, very small and hardly exceeds the limits of experimental error. The splitting of benzoylisoglutamine by natural papain is considerably faster. In

TABLE II

Activation of Papain after Oxidation with Iodine

Substrate	Enzyme No.	Hydrolysis in ml. of 0.01 N KOH						
		1 hr.	2½ hrs.	5 hrs.	8 hrs.	24 hrs.	48 hrs.	120 hrs.
Gelatin	I	0.73				1.49		
	II	0.00				0.17		
	III	0.64				1.22		
Benzoylisoglutamine	I		0.62			0.93		
	II		0.02			0.10		
	III		0.50			0.67		
Hippurylamide	I			0.54		0.96		
	II			0.00		0.01		
	III			0.30		0.75		
Carbobenzoxyglutamylglycylglycine	I				0.44	0.86		
	II				0.00	0.02		
	III				0.16	0.43		
Diglycyl-*l*-leucylglycine	I						0.47	0.73
	II						0.00	0.00
	III						0.07	0.20
Triglycyl-*l*-leucylglycine	I					0.53		1.19
	II					0.00		0.00
	III					0.23		0.78

Enzyme I represents HCN-papain; Enzyme II, natural papain after iodine treatment; Enzyme III, natural papain after treatment with iodine and then with HCN. Considering the different concentrations of Enzymes I, II, and III, there was used for the individual experiments 0.2 ml. of Enzyme I, 0.25 ml. of Enzyme II, or 0.33 ml. of Enzyme III. Carbobenzoxyglutamylglycylglycine was dissolved in the equivalent amount of 0.1 N NaOH. The other details of these experiments are the same as those in Table I. (The preparation of carbobenzoxyglutamylglycylglycine will be described in a later communication.)

this case, one may attain a high splitting without difficulty and may also isolate the splitting products (benzoylglutamic acid and ammonia). These are the same products as those obtained with HCN-papain. The action of HCN on papain therefore effects

no change in specificity with benzoylisoglutamine, but does effect an increase in the amount of active enzyme and, accordingly, an acceleration of the splitting of the substrate.

In Table II experiments involving the influence of iodine on the papain digestion of a number of synthetic substrates are described. There are included two free polypeptides which have been found, through experiments carried on in cooperation with Dr. Joseph S. Fruton, to be hydrolyzed with papain. From Table II it may be seen that the inactivation of papain with iodine extends not only to the splitting of gelatin and acylated peptides, but also to the splitting of free peptides.

One finds reported in the literature that oxidized papain can be completely reactivated with reducing agents. In our experiments with iodine, this goal could not be reached. The inactivation was undertaken with the minimum amount of iodine, a condition under which the enzyme could be reactivated with HCN towards all the substrates investigated.[1] However, the regenerated activity was always less than that of the original papain after activation.[2] Although the same reactivated enzyme preparation was used in all cases, the loss in activity was more pronounced in the case of substrates with a smaller splitting rate. Apparently, this is due to the presence of inhibitive substances formed in consequence of the treatment with iodine.

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EXPERIMENTAL

Natural Papain—225 mg. of powdered papain were shaken for 1 hour with 15 ml. of water; the solution was filtered, combined with 25 ml. of citrate buffer, pH 5.0, and diluted with water to 50 ml.

HCN-Papain—To a solution of 225 mg. of papain in 15 ml. of water were added 25 ml. of citrate buffer, pH 5.0, and 10 ml. of

[1] It appears possible that the inactivation of papain by hydrogen peroxide is also reversible with regard to synthetic substrates, when performed with the minimum amount of hydrogen peroxide.

[2] Maschmann and Helmert (7) have found, in experiments with gelatin, that the inactivation of papain with oxygen in the presence of cysteine-heavy metal was only partially reversible.

1.2 per cent HCN. The solution was warmed to 40° for 2 hours before being used.

Iodine-Inactivated Papain—0.05 N iodine in aqueous KI was added dropwise, with shaking and cooling, to 40 ml. of natural papain (as above) until the first faint yellow coloration occurred. This point was reached after the addition of about 2 ml. After the oxidized solution had been allowed to stand another hour at room temperature, 1 ml. of 0.1 N NaOH was added and the solution made to 50 ml. with water.

Papain-HCN Reactivated after Iodine Oxidation—35 ml. of iodine-inactivated papain solution were combined with 15 ml. of 1.2 per cent HCN and maintained for 2 hours at 40° before use.

0.2 ml. of either of the first two enzyme solutions corresponds to 0.25 ml. of iodine-inactivated or 0.33 ml. of the reactivated enzyme solution.

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