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The Inactivation of a Bacteriophage by a Component of Papain

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It was found by Kalmanson & Bronfenbrenner (1943) that papain could reactivate phage PC which had been inactivated by antiserum and that the phage itself was unaffected by the enzyme. When this experiment was repeated with 'phage 3' in this Laboratory it was found that the phage was greatly inactivated. During an investigation of the mode of inactivation of this phage by papain the work of Kozloff, Lute & Henderson (1957) was published. These authors included crystalline papain in a list of agents which could inactivate coliphages T2 and T4 by damaging the tail tip, a part of the phage which must be intact for adsorption to host cells to occur. The agents they used had in common the property, recently described in papain by Johnston (1956), of breaking thiolester bonds. Kozloff *et al.* (1957) concluded that papain damaged the phage by breaking thiolester bonds which normally were responsible for the integrity of the tail in the active phage particle. That papain acts on the tails of both phage T4 and phage 3 seems adequately proven since both these phages require tryptophan for adsorption and both (Kozloff *et al.* 1957; Fildes & Kay, 1959) require tryptophan to be inactivated by papain. As adsorption involves only the tip of the phage tail then presumably papain attacks that part of the phage. However, as shown in this paper, the action of papain on phage 3 is not due to its thiolesterase activity.

MATERIALS AND METHODS

Bacteriophage and host bacteria. Phage 3 and its host *Bacterium coli* 518(3) have been described by Fildes & Kay (1959). The phage was isolated from Oxford sewage and, like the coliphages T2, T4 and T6, was found to contain the unusual pyrimidine hydroxymethylcytosine. It exists in two mutant forms, one of which requires tryptophan for adsorption to the host, whereas the other is largely independent. The latter was used in the work described in this paper. Stocks of the phage were prepared in an ammonia–glucose medium and purified by differential centrifuging as described by Kay (1959).

Measurement of the antiphage activity of papain. The papain preparations for testing were always made up in a buffer containing KH_2PO_4–Na_2HPO_4, pH 7·6, 0·033 M with respect to phosphate. This buffer is referred to as phosphate buffer in this paper. Serial dilutions (ratio 2:3 over the centre of the active range) of papain in this buffer were mixed with standard amounts of phage 3 (5×10^6/ml.) and incubated at 37° for 30 min. The total volume was 10 ml. DL-Tryptophan (0·1 mM) was added to enable the reaction between phage and papain to proceed at maximum rate, even though the phage was a mutant that was virtually independent of tryptophan. The effect of tryptophan on the action of papain on phage 3 was discussed by Fildes & Kay (1959). Controls without papain were set up and assayed for phage, as described by Fildes & Kay (1957), at the beginning, middle and end of the experiment and averaged to give the input. All the tubes were assayed for phage at 30 min. and the percentage inactivation was plotted against concentration of papain. The amount of papain required to destroy 50% of the input phage was calculated from the curves. Typical curves are given in Fig. 4.

Measurement of thiolesterase activity. Benzoylglycine thiol ethyl ester (BGTEE) was used as a substrate for thiolesterase. It was synthesized from benzoylglycine by the method described by Johnston (1956). In order to permit the thiolesterase of papain to act on BGTEE under conditions as similar as possible to those in which papain was tested against phage the tests were made in phosphate buffer (0·033 M), pH 7·6. The standard input of BGTEE was 5 μmoles in a volume of 1·5 ml. Methanol (10%, v/v) was included to maintain the BGTEE in solution. The papain to be tested, already in phosphate buffer, was diluted appropriately in the same buffer and added to the BGTEE. Thiolester was determined immediately and after 30 or 120 min. at 37° by the hydroxamic procedure of Lipmann & Tuttle (1945). The resulting coloured mixtures were centrifuged to remove the protein precipitates. The extinctions of the supernatants were measured in a Hilger Spekker absorptiometer with filters with a transmission maximum at 550 mμ. The extinction bore a linear relationship to the amount of BGTEE up to at least 8 μmoles.

Electrophoresis. Zone electrophoresis in a starch-grain bed 8 cm. × 30 cm. × 1 cm. deep at 4° was performed by a method similar to that described by Kunkel (1954). Potato starch (British Drug Houses Ltd.) was first washed in water and then equilibrated against three changes of the buffer chosen for the experiment. The sample of papain to be examined was incorporated into the starch bed near the anode end as it was known that all the components were positively charged at the pH chosen. Electrophoresis was carried out at 3–4 v/cm. as measured across the starch bed. The movement of the band was followed by taking filter-paper prints of the surface of the starch, drying the paper and staining in ethanolic 0·1% bromophenol blue, containing 4% (w/v) of mercuric chloride, for 1 min. The positions of the protein bands were revealed on washing the strips in 1% (v/v) acetic acid. When the bands had travelled

a suitable distance the starch bed was cut into sections and the protein extracted by suspension of the starch in 5 ml. of phosphate buffer followed by centrifuging and removal of the supernatant. This procedure was repeated twice; the extracts were pooled and finally filtered through Whatman paper no. 1 to remove the remaining starch grains. Microbial contamination was prevented by inclusion of a small crystal of thymol in the extracts.

Nitrogen determination. The protein-nitrogen content of the various samples studied was determined by the micro-Kjeldahl procedure of Campbell & Hanna (1937). A correction occasionally had to be made for nitrogen contributed by traces of ammonium sulphate remaining in fractions prepared by the use of this material.

Crude papain. All the papain used was obtained in a single batch from British Drug Houses Ltd. It was a fine light-brown powder and was presumably pulverized dried latex.

RESULTS

Antiphage activity of crude-papain extracts. Extracts of crude papain in phosphate buffer were tested against phage 3 at a wide range of dilutions. The phage and papain were allowed to react for 30 min., after which the amount of phage remaining was determined. A typical result is given in Fig. 1. It shows that the highest concentration of papain has little effect on the phage but that as the concentration is decreased the amount of phage destroyed rises to a maximum, when 93 % is lost, and then falls again. All extracts of papain, whether made in water, phosphate buffer or cysteine solution (provided that the last-named extract was dialysed before testing), showed this same type of curve, which suggests that they contained two active components, an inhibitor which was diluted out first and an antiphage material which was still active at high dilutions.

Effect of various agents on the antiphage extracts of papain. During the course of experiments to decide whether the proteolytic enzyme of papain was involved in the antiphage action it became clear that such agents as cysteine, ethylenediaminetetra-acetic acid (EDTA) and citrate, which activate the protease, inhibited the antiphage activity of papain. Cysteine, depending on the concentration relative to that of the papain, could block the antiphage component and the hypothetical inhibitory component. In Fig. 1 are two curves showing the effect of cysteine. For one, papain was titrated against phage in the presence of constant (0·033 M) cysteine. The antiphage activity was reduced but the inhibitory effect was also diminished since more phage was lost in the highest papain concentration with cysteine than without. For the second curve, papain and cysteine at an initial concentration of 0·025 M were diluted together. The antiphage activity was largely unaffected and was not suppressed at high concentrations of papain. It appears therefore that a certain ratio of cysteine to papain can block the hypothetical inhibitor of antiphage but that higher ratios block the antiphage as well. The effect of citrate and EDTA on the antiphage activity of papain is shown in Fig. 2. The amount of papain, in this case an ammonium sulphate fraction, was that which gave the maximum inhibition of phage when tested in phosphate buffer. This was kept constant while the citrate and EDTA were varied. The

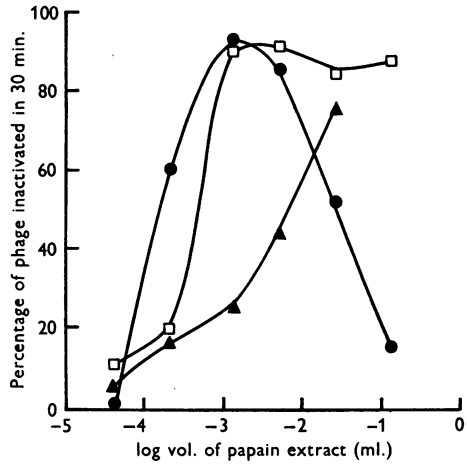

Fig. 1. Inactivation of phage 3 by papain. An extract of 4 g. of papain in 100 ml. of phosphate buffer (0·033 M), pH 7·6, was tested in fivefold serial dilutions against phage 3 (5×10^5/ml.) at 37° for 30 min. in phosphate buffer (●), in the presence of constant (0·033 M) cysteine (▲) and in cysteine diluted with the papain from 0·025 M (□).

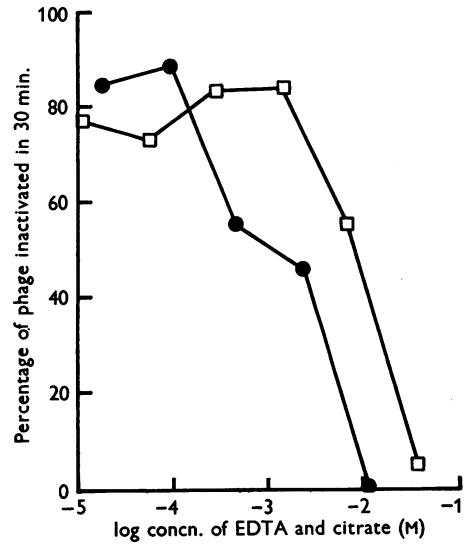

Fig. 2. Effect of citrate (□) and EDTA (●) on the inactivation of phage 3 by a constant amount of papain.

amount of phage inactivated is plotted against the concentration of these substances. Both EDTA and citrate abolish the antiphage activity if added in the appropriate concentration, EDTA being rather more active than citrate. The three agents tested above, cysteine, EDTA and citrate, have in common the property of sequestering metal ions and their action suggests that some metal is an essential component of the antiphage system. All the tests have been done in 0·033 M-phosphate buffer, and it is known that this, too, is capable of binding some metal ions and effectively removing them from the system. It was therefore of importance to test whether phosphate itself was affecting the antiphage activity of papain, and for this purpose two sets of reactants, phage, tryptophan and papain, were prepared, one in the usual phosphate buffer and the other in 0·033 M-sodium chloride. Dilutions of papain were then tested against phage, with the results given in Fig. 3. The antiphage activity of papain is greatly reduced in the absence of phosphate, which suggests that some inhibitory ions normally present in the reaction mixture are sufficiently reduced in concentration by the phosphate to permit the papain to inactivate the phage. However, taking other observations into account, together with the inhibitory effect of citrate etc., it appears that a particular balance between the papain and metal ions must be set up for papain to exert its maximum destructive effect on phage. It has not yet been possible to clarify this situation and to determine what metals are involved in the papain–phage system, and since the main object of this paper is to consider the thiolesterase of papain and its role in phage-inactivation the matter of the metals and hypothetical inhibitor will not be examined further.

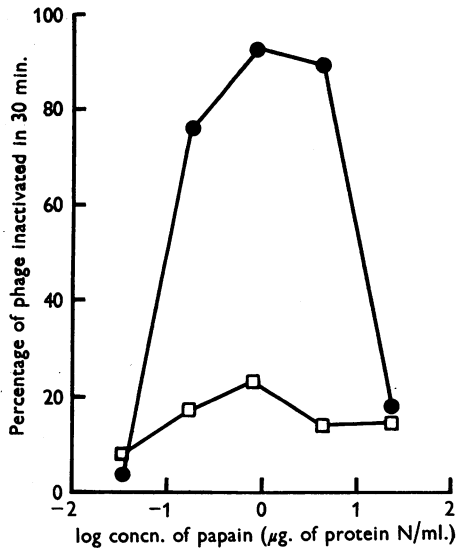

Fig. 3. Effect of phosphate on the antiphage activity of papain. An extract of papain was tested in fivefold dilutions against phage 3 in phosphate buffer (0·033 M) (●) and in NaCl (0·033 M) adjusted to pH 7·6 (□).

Effect of crystalline papain on phage. Papain was crystallized by the method of Kimmel & Smith (1954), starting from dried powdered latex. In this procedure an extract of latex in 0·04 M-cysteine is prepared. This is adjusted to pH 9·0 to remove an inactive component, treated with 0·4 saturated ammonium sulphate to precipitate the protease, which is then crystallized from 0·02 M-cysteine solution. After two recrystallizations from sodium chloride solution the crystals were centrifuged down, dissolved in a little water and dried from the frozen state and stored over phosphorus pentoxide. The antiphage activity of this material dissolved in phosphate buffer was tested by the standard procedure, and the results, together with those of the solution before crystallization and the combined mother liquors after two recrystallizations, are shown in Table 1. It is clear that the antiphage activity is not in the crystalline material.

Thiolesterase of crystalline papain. Extracts of crude-papain latex and crystalline mercuripapain have been found by Johnston (1956) to hydrolyse the thiolester bonds of BGTEE and thiol *iso*propyl ester. The crystalline papain as prepared above and found to be inactive against phage was then tested for thiolesterase against BGTEE. Thiolesterase activity was found, as shown in Table 2.

Table 1. *Inactivation of phage* 3 *by fractions of papain*

Ammonium sulphate fraction before crystallization		Protease fraction (twice recrystallized)		Mother liquors	
Protein N (μg./ml.)	Inactivation (%)	Protein N (μg./ml.)	Inactivation (%)	Protein N (μg./ml.)	Inactivation (%)
53·4	17	450	0	25·6	36
10·7	49	90	0	5·1	76
2·1	57	33·4	0	1·0	25
0·43	32	6·7	0	0·2	2
0·85	4	1·3	0	0·04	0

Fractionation of papain. As it had been shown that the crystallizable component of papain did not contain the antiphage activity but did have thiolesterase activity, doubts arose as to the likelihood of the antiphage component having thiolesterase activity, although this was still a possibility. It was therefore decided to fractionate the ammonium sulphate-soluble part of the cysteine extract of latex which had been discarded during the isolation of crystalline papain. Further fractions were prepared by raising the ammonium sulphate concentration to 50, 70 and 100% saturated. Antiphage activity and thiolesterase were found in all of these fractions but were most marked in the 50% and 70% saturated ammonium sulphate precipitates. However, it became obvious that the separation of thiolesterase and antiphage activity was not feasible by this procedure, and so another method, electrophoresis, was tried. A preliminary test, in which the 40–70% saturated ammonium sulphate fraction was examined in the Antweiler microelectrophoresis apparatus, showed that at pH 5·0, 7·0 and 9·0, in acetate, phosphate and borate buffers (0·02 M with respect to these ions), the papain divided into several components, all of which were positively charged. The separation appeared to be most complete at pH 5·0, and so it was decided to fractionate papain by preparative electrophoresis at this value. The starch-grain-bed procedure was adopted. The starting material was a 40–70% saturated ammonium sulphate precipitate prepared from the mother liquors left after removal of the 40% saturated ammonium sulphate precipitate in the Kimmel & Smith (1954) procedure for crystallizing the protease. The precipitate was redissolved in sodium acetate–acetic acid buffer, pH 5·0 (0·02 M-acetate), and incorporated (22 mg. of protein nitrogen) into the starch layer. After electrophoresis at 3–4 v/cm. for 24 hr. it was found that the leading edge of the protein band, which was about 8 cm. long, had reached to within 1 cm. of the end of the starch bed and was divided into a sharp leading band followed by a second diffuse band. The protein bands were then removed and cut into four sections, one of which contained the

Table 2. *Hydrolysis of benzoylglycine thiol ethyl ester by crystalline papain*

Input of thiolester was 5 μmoles in a total volume of 1·5 ml. of phosphate buffer (0·033 M), pH 7·6, containing 10% (v/v) of methanol. The mixture was kept at 37° for 30 min.

Crystalline papain (μg. of protein N/ml.)	Thiolester hydrolysed (% of input)
38·0	96·5
19·0	75·0
9·5	35·5
4·8	17·5
None	7·0

Table 3. *Electrophoretic fractionation of papain at* pH 5·0

The fraction of papain soluble in 40% saturated ammonium sulphate but precipitated by 70% saturated ammonium sulphate was subjected to electrophoresis in starch in acetate buffer, pH 5·0. The protein band was divided into four parts, numbered 1 to 4 from the anode end, and tested for antiphage activity and for thiolesterase activity.

Fraction tested	Protein N in test (μg./ml.)	Thiolester hydrolysed (%) 30 min.	Thiolester hydrolysed (%) 120 min.	Protein N to inactivate 50% of phage (μg./ml.)
40–70% Ammonium sulphate ppt.	248	100	—	0·08
	124	96	—	
	49	60	—	
	32	—	60	
Fraction 1	67	—	0	7·5
	50	0	—	
	25	0	—	
	10	0	—	
Fraction 2	148	—	100	0·80
	111	96	—	
	56	64	—	
	33	18	—	
Fraction 3	110	—	100	0·035
	82	86	—	
	41	53	—	
	24	8	—	
Fraction 4	32	—	0	0·0045
	24	0	—	
	12	0	—	
	7	0	—	

well-defined frontal band and the rest were equal parts of the remaining diffuse band. They were numbered 1 to 4 from the anode end. The protein in each fraction was extracted with three lots of 5 ml. phosphate buffer, pH 7·6 (0·033 M), by centrifuging and tested for protein-nitrogen content, antiphage activity and thiolesterase activity. The results are given in Table 3 and Fig. 4.

Fig. 4 shows the relationship between protein-nitrogen concentration and phage inactivated in 30 min. From it the amounts of protein nitrogen required to destroy 50 % of the phage in 30 min. were obtained and included in Table 3. It appears that all fractions contained some antiphage activity, but that this lay predominantly in fraction 4, by comparison with which the others had small activities in the approximate ratio 1600:180:8:1 in the amounts of protein nitrogen required to inactivate 50 % of the phage. Thiolesterase was found only in fractions 2 and 3. None could be detected in fraction 4 even at the highest concentration and longest time (2 hr.) tested. It was not possible to lengthen the duration of the test owing to the instability of the thiolester, which decreased by 12–15 % in 2 hr. in the controls. Nevertheless, the above results show that papain could be divided by electrophoresis into one fraction (no. 4) which contained a very active antiphage and no thiolesterase and other fractions which contained thiolesterase and antiphage material of a much lower activity. This is taken to mean that papain contains an antiphage component which is not a thiolesterase. Further electrophoretic fractionations were made at pH 7·6 and 4·0. At pH 7·6, which was maintained in 0·02 M-phosphate buffer, a less-satisfactory separation of antiphage activity of thiolesterase was obtained. No clearly defined front band was observed, but on extraction from the starch the material at the front of the diffuse protein band showed the highest antiphage activity and the lowest thiolesterase activity. The

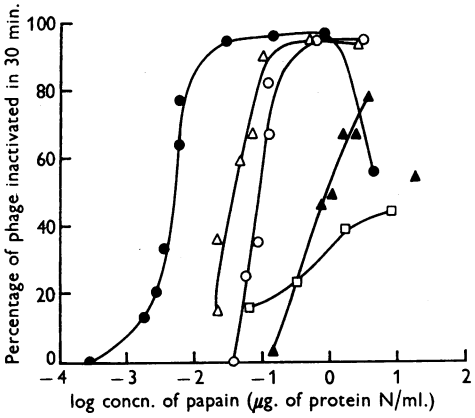

Fig. 4. Antiphage activity of a 0·4–0·7 saturated ammonium sulphate fraction of papain (○) compared with that of fractions 1 (□), 2 (▲), 3 (△) and 4 (●), prepared at pH 5·0 by zone electrophoresis in starch.

Table 4. *Electrophoretic fractionation of papain at* pH 4·0

The fraction of papain soluble in 40 % saturated ammonium sulphate but precipitated by 70 % saturated ammonium sulphate was subjected to electrophoresis in starch in formate buffer, pH 4·0. The protein band was divided into four parts numbered 1 to 4 from the anode end and tested for antiphage activity and for thiolesterase activity.

Fraction tested	Protein N in test (μg./ml.)	Thiolester hydrolysed in 120 min. (%)	Protein N to inactivate 50 % of phage (μg./ml.)
40–70 % Ammonium sulphate ppt.	32	88	0·05
	19	46	
	13	26	
	6	11	
Fraction 1	73	41	2·5
	32	4	
	19	0	
	13	0	
Fraction 2	96	100	1·0
	32	34	
	19	0	
	13	0	
Fraction 3	32	0	0·05
	19	0	
	13	0	
Fraction 4	45	0	0·016
	32	0	
	19	0	
	13	0	

separation at pH 4·0 was performed in sodium formate–formic acid buffer (0·02 M-formate). Again a clear separation of antiphage and thiolesterase activity was obtained. The main protein band was preceded by a minor but well-defined band. This proved on extraction to contain the most active antiphage material and, as at pH 5·0, no thiolesterase. The results are given in Table 4.

Effect of cysteine on thiolesterase and on phage 3. Johnston (1956) observed that cysteine, when used in an attempt to activate the thiolesterase of crystalline papain, was itself able to destroy the benzoylglycine thiol ethyl ester used as a substrate. This result has been confirmed during the present work, as is shown in Table 5. Cysteine in varying amounts was incubated with BGTEE (3·4 μM) in phosphate buffer (0·033 M), pH 7·6, containing methanol (10 %, v/v) to maintain the BGTEE in solution. After 30 min. at 37° the amount of BGTEE remaining was determined. It can be seen that in an equimolar mixture of cysteine and BGTEE, 57 % of the BGTEE has been lost by reaction with the cysteine. Larger amounts of cysteine destroy 82 % of the BGTEE in 30 min. The reaction, which is of a type described by Strecker, Mela & Waelsch (1955), is said to involve a sulphur-to-sulphur transfer of the acyl amino acid group of the thiol ethyl ester to the thiol group of cysteine, followed by a rearrangement during which the acyl group migrates to the nitrogen of the cysteine. The *N*-acyl cysteine does not form a hydroxamic acid under the conditions of the analysis.

The activity of phage 3 was not affected by treating for 30 min. at 10^5 particles/ml. in 0·033 M-phosphate buffer, pH 7·6, with cysteine varying from 0·05 to 0·002 M.

DISCUSSION

The results described confirm the discovery of Johnston (1956) that papain contains a component capable of splitting the thiolester bonds of benzoylglycine thiol ethyl ester. This component, known as a thiolesterase, occurs in the crystallizable protease fraction as isolated by the procedure of Kimmel & Smith (1954) and is probably identical with it. Kozloff *et al.* (1957) found that a commercially produced crystalline papain could inactivate coliphages T2 and T4 under conditions in which the thiolesterase would be active but the protease would be inactive. They believed that the papain inactivated the phages by breaking thiolester bonds in their tails. In an attempt to confirm this result with phage 3, which is somewhat similar to T4, it was found that whereas crude papain caused rapid inactivation, crystalline papain was without effect. The antiphage activity resided in the fractions of papain discarded during crystallization of the protease. Zone electrophoresis in starch showed that the majority of the antiphage activity travelled as an isolated band ahead of the main protein band. The material in this band was found to possess powerful antiphage activity but indetectable thiolesterase activity. It is concluded therefore that the thiolesterase is not responsible for the action of papain on phage 3. Presumably thiolester bonds, if they exist in phage 3, are not available to the thiolesterase of papain nor are they susceptible to the action of cysteine, which rapidly destroys thiolester bonds in BGTEE but has no effect on the activity of phage 3. The antiphage component of papain is being investigated further.

The principal difference between the results of Kozloff *et al.* (1957) and those described in the present paper is that whereas the former support the view that papain inactivates phage T2 by breaking thiolester bonds the latter show that the inactivation of phage 3 by papain is unlikely to be due to its thiolesterase. At present it has not been resolved whether this difference is due to different phages or to different experimental conditions.

Table 5. *Destruction of thiolester by cysteine*

Constant amounts of BGTEE (3·4 mM) were incubated at 37° in phosphate buffer (0·033 M), pH 7·6, with the indicated amounts of cysteine in a total volume of 1·5 ml., including 10 % (v/v) of methanol.

Cysteine (mM)	Thiolester lost in 30 min.
33	90
13	91
6·7	90
3·4	66
1·3	34
0·67	25
None	9

SUMMARY

1. A bacteriophage, phage 3, originally isolated from Oxford sewage, is inactivated by dilute extracts of papain.

2. The inactivation is blocked by cysteine, citrate and ethylenediaminetetra-acetic acid, which suggests that a metal might be involved in the system.

3. The protease of papain, when crystallized by the procedure of Kimmel & Smith (1954), was found to possess thiolesterase activity as shown by Johnston (1956).

4. The thiolesterase has no effect on phage 3 although Kozloff *et al.* (1957) have found that the somewhat similar phages T2 and T4 are inactivated by the thiolesterase of crystalline papain.

5. The antiphage component of crude papain has been separated by zone electrophoresis and found to have no thiolesterase activity.

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Parapepsinogen II: The Zymogen of Parapepsin II

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The isolation and characterization of two new enzymes, parapepsins I and II, from crude porcine pepsin has already been described (Ryle & Porter, 1959).

In seeking to determine whether these enzymes might be derived from pepsinogen or whether they arose from other precursors partly purified extracts of porcine fundic mucosa have been chromatographed on diethylaminoethylcellulose and a zymogen for parapepsin II has been found. The isolation and some of the properties of this zymogen are described in this paper.

EXPERIMENTAL

Abbreviations. The following abbreviations will be used throughout this paper: diethylaminoethyl, DEAE; haemoglobin, Hb; acetyl-DL-phenylalanyl-L-di-iodotyrosine, APD; 1-fluoro-2:4-dinitrobenzene, FDNB; dinitrophenyl, DNP.

Instruments. Extinction coefficients were determined with either the Hilger Uvispek or a Unicam spectrophotometer.

Determinations of pH were made either with an E.I.L. direct-reading pH meter or a Cambridge pH meter, the meters being standardized against buffer solutions made from Cambridge buffer tablets.

Buffer solutions. Except where otherwise stated buffer solutions were made from sodium salts. Their concentrations are stated with respect to the anionic component.

Activity determination with haemoglobin. Assays of activity against haemoglobin were performed in duplicate by a method based on that of Northrop, Kunitz & Herriott (1948) as described by Ryle & Porter (1959). In the earlier part of the work, when zymogens were assayed 0·1 ml. of the zymogen solution in 0·02 M-phosphate buffer, pH 6·9, was mixed with 0·1 ml. of 0·035 N-HCl and allowed to stand at 35·5° for 10 min. to allow activation to occur before addition of 1 ml. of acid-denatured haemoglobin (2%, w/v). Later it was found that no significant difference appeared in the assay of either pepsinogen or parapepsinogen II if this activation period were omitted, and 0·2 ml. samples were then assayed by the addition of 1 ml. of acid-denatured

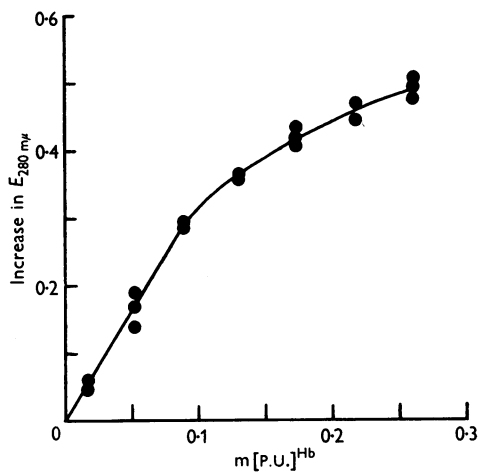

Fig. 1. Standard curve for parapepsin II. A solution (0·2 ml.) of enzyme in 0·1 M-acetate, pH 5·6, was incubated with 1 ml. of acid-denatured haemoglobin (4 vol. of 2·5% Hb + 1 vol. of 0·39 N-HCl) for 10 min. at 35·5°. The reaction was stopped with 5 ml. of 4% (w/v) trichloroacetic acid. Units were chosen as described in the text.

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