BBA 65579

THE ACTIVATION REACTION OF PAPAIN

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(Received October 6th, 1966)

SUMMARY

1. Papain is reversibly inactivated in the presence of air and low concentrations of cysteine. This inactivation is enhanced by Fe^{2+} and Cu^{2+} and is retarded by EDTA. In the absence of cysteine, active papain (separated from the activators by gel filtration) is inactivated at a much lower rate in an almost irreversible manner.

2. Papain inactivated in the presence of cysteine has exactly the same rate of activation as papain which is inactive after its preparation according to Kimmel and Smith. It is concluded that these papains are identical.

3. Experiments with radioactively labelled cysteine have shown that reversibly inactivated papain is a mixed disulphide of papain and cysteine. This cysteine is removed on activation.

4. Some experiments with divalent metal ions have shown that Cd^{2+} has a strong, Zn^{2+} a very strong preference for inhibitory combination with papain over binding with cysteine. Fe^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+}, show no or a small preference for papain over cystein. On the other hand, in the absence of cysteine, Zn^{2+} is removed from papain by EDTA, but not Hg^{2+}.

INTRODUCTION

It has been known for a long time that papain is activated by compounds like cysteine, sulphide, sulphite *etc.* A review of these older results has been made by Smith[1]. Optimum activation was found to occur on the simultaneous application of a thiol compound like cysteine and a heavy metal-binding agent like EDTA (ref. 2), or by application of BAL (ref. 3), a compound which combines both functions of a thiol compound and a metal binder. Furthermore Finkle and Smith[4] were able to show that papain, after activation, has a thiol content of 0.3 to 0.8 groups per molecule, proportional to the activity of the particular preparation. This proportionality between activity and thiol content has been confirmed by Sanner and Pihl[5].

Two matters are as yet unknown: (1) the nature of the activation reaction, (2) the reason why different preparations of papain after activation have a different

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Abbreviations: BGEE, benzoylglycine ethyl ester; BAEE, benzoylarginine ethyl ester.

thiol content and hence a different activity although these preparations are indistinguishable in other respects.

This paper will deal mainly with the first question. It will be shown that the activation reaction of papain, prepared according to KIMMEL AND SMITH, consists of the removal of a half-cystine residue from the enzyme molecule and the concomitant liberation of the papain thiol group. Furthermore, some attention will be given to the role played by EDTA in attaining optimum activation.

EXPERIMENTAL

The equipment for automatic titration, the general assay procedures and the materials were those described in an earlier paper[6]. The divalent metal salts were commercial preparations of *pro analysi* quality.

Assay conditions were 0.02 M BAEE, 0.3 M KCl, 25°, pH 6.0 for the experiments of Figs. 1–3, and 0.03 M BGEE, 0.3 M KCl, 37°, pH 6.0 for those of Tables II and III.

Gel filtration of 5-ml samples was carried out at room temperature (about 20°) on 2.0 cm × 20 cm columns of Sephadex G-25 fine (Pharmacia, Uppsala, Sweden). The elution rate was adjusted to 38 ml/h, utilizing a Miniflow precision micropump, Type 4501, 3× (L.K.B. Produkter, Stockholm, Sweden). Fractions were cut every 4 min. The elution buffer was 0.02 M acetate–acetic acid (pH 5.0). The column and elution buffer contained 1 mM EDTA when the enzyme sample was to be used for thiol titration. The complexing agent was omitted when the sample was to serve for inactivation experiments. In a few experiments, traces of undesirable reducing groups in the Sephadex columns were removed by oxidation: 5 ml of water containing 0.05 M KI and 0.01 M I_2 were introduced into the column. The brown band was eluted with 0.05 M KI and the latter solution was finally replaced by the acetate buffer.

Example of an activation experiment. A sample of 0.5 g papain gel (approx. 10% protein), prepared according to KIMMEL AND SMITH[7], was dissolved in 3.5 ml of 0.02 M acetate (pH 5.0), 0.5 ml of 0.01 M EDTA and 0.5 ml of 0.05 M cysteine–HCl which had been previously adjusted to about pH 6. The reaction mixture was stored at room temperature for 10 to 15 min and subjected to gel filtration.

Thiol titration. For amperometric argentometric titration in imidazole buffer (pH 7.0) (ref. 8), 1-ml aliquots of activated papain were used in a total volume of 3 ml of titration mixture. Duplicate titrations were carried out in the absence and in the presence of 2 mM Na_2SO_3 in order to check completeness of the activation. One sample of activated papain was analyzed both by amperometric titration and by the colorimetric method of ELLMAN[9]. These methods gave identical results. The thiol content of a number of papain preparations varied within the narrow limits of 0.50 and 0.63 groups per molecule.

Example of an inactivation experiment, with labelled cysteine. A 4.0-ml aliquot, containing about 0.7% of activated and gel-filtered papain, 1.8 ml of 10 mM L-cysteine and 0.09 M imidazole (pH 7.0) was mixed with 0.1 ml (10 μC) of L-[^{35}S]cysteine solution (Radiochemical Centre, Amersham) and adjusted to pH 7.0. After 30 min at 5°, 0.1 ml of $6 \cdot 10^{-3}$ M $FeSO_4$ was added and the mixture stored at 5° for 1–2 days. One 0.1-ml aliquot was then used to check the inactivation and three 0.1-ml aliquots were used for counting in 3-fold. The remaining part was subjected to gel filtration at pH 5.0. Of each eluent fraction, 0.3 ml was used for ultraviolet absorption measurement at 280 mμ and 0.2 ml for counting. In the second gel filtration run (*cf.* Fig. 3B) 0.4-ml aliquots were used for counting.

The aliquots to be counted were absorbed in lens-tissue discs of 2 cm diameter on metal discs. A low-activity low-background Geiger-Müller counter (Philips Type 18536), an automatic sample changer and a digital print-out served for the measurements. The counting time was 10–20 min per sample. For calculations, a molecular weight of papain of 22 000 and an ultraviolet absorption at 280 mμ of $E^{1\%}_{1\,\mathrm{cm}} = 25$ were assumed.

The conditions of reversible inactivation

Papain is generally prepared in six fractionation steps according to the method of KIMMEL AND SMITH[7]. Cysteine is added in Steps I, IV and V. One might therefore expect papain preparations to be active even in the absence of a reducing agent. Indeed, SANNER AND PIHL[5] found such activity (from 30 to 60%) after taking some precautions against heavy-metal contamination during the preparation of papain. The present author had similar results when preparing papain entirely according to KIMMEL AND SMITH. Different preparations had 'spontaneous' activities (*i.e.* activity in the presence solely of 1 mM EDTA) of zero to about 30% of 'optimum' activities (*i.e.* activity in the presence of both 5 mM cysteine and 1 mM EDTA).

The most likely cause of papain inactivity, in spite of the presence of cysteine during the fractionation procedure, is air oxidation. Experiments were therefore carried out on the stability of papain in the presence of air. Papain was activated with cysteine and EDTA, separated from these compounds on a Sephadex column and stored in the refrigerator at 5°, at pH 7.0. The enzyme was found to be remarkably stable (Fig. 1, Curve A). Only extended storage of a more dilute papain solution led to loss of spontaneous activity (Fig. 2, Curve B). Furthermore, most of the activity was lost in an irreversible manner, since the optimum activity, too, had greatly suffered (Fig. 1b).

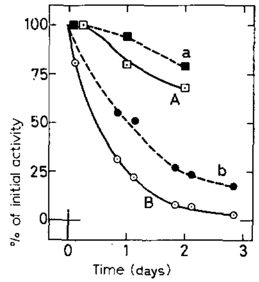

Fig. 1. Inactivation of activated papain in the absence of activator at pH 7.0, 5°. Open symbols, spontaneous activity; closed symbols, optimum activity, in percentage of initial values. A, $1.5 \cdot 10^{-4}$ M papain; B, $2.3 \cdot 10^{-5}$ M papain.

Quite a different picture was obtained when the conditions of the last step of the preparation of papain (Step VI) were imitated. Since cysteine (20 mM) is present in Step V and the resulting precipitate is a voluminous one, the solution of Step VI (which is stored overnight at 5°) also contains some cysteine (1–3 mM). Therefore the experiments of Fig. 1 were repeated in the presence of low concentrations of cysteine.

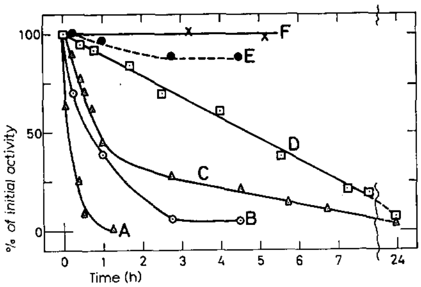

Fig. 2. Inactivation of activated papain in the presence of cysteine at pH 7.0, 5°. Open symbols, spontaneous activity; closed symbols, optimum activity. A, $2.4 \cdot 10^{-5}$ M papain, $2.0 \cdot 10^{-4}$ M cysteine; B, $1.6 \cdot 10^{-4}$ M papain, $1.0 \cdot 10^{-3}$ M cysteine; C, $1.6 \cdot 10^{-4}$ M papain, $3.0 \cdot 10^{-3}$ M cysteine, 10^{-4} M Fe^{2+}; D, like C, without Fe^{2+}; E, like B; F, like B, in addition 10^{-3} M EDTA.

The most relevant data are given in Fig. 2. There is a rapid loss of spontaneous activity (Curve B) which is even more rapid at lower cysteine concentrations (Curve A) and less rapid at higher cysteine concentration (Curve D). EDTA retards the inactivation (Curve F), indicating an enhancing effect of contaminating heavy metals. This latter effect is confirmed by the enhancing effect of added Fe^{2+} (*cf.* Curves C and D) and an even stronger effect of Cu^{2+} (not shown). Furthermore, in sharp contrast to the results in Fig. 1, Fig. 2 (Curve E) shows that there is irreversible inactivation in cysteine solution only to a slight extent. This may be greater only when the inactivation is very rapid: a decrease in optimum activity of 30% in the experiment of Fig. 2, Curve A, and a decrease of 40–50% in a few cases of Cu^{2+}-catalyzed inactivation were observed.

The rapidity of the inactivation and its reversibility make it likely that the papain inactivated artificially under the conditions of Fig. 2 is identical to papain inactivated of its own accord during the preparative procedure. This identity could be confirmed on the strength of the rate of activation of both kinds of inactive papain by cysteine. The rate of activation was measured with the aid of a method described in a previous paper[10], in the presence of substrate. Under two sets of conditions, the two types of inactive papain gave identical results (Table I). On the other hand, a sample

TABLE I

ACTIVATION RATE OF INACTIVE PAPAIN PREPARATIONS

	Conditions of reactivation		
	BAEE concn. (mM)	*Cys concn. (mM)*	*k* (*min*$^{-1}$)
Inactive on preparation	120	5	$0.55^{4} \pm 0.018$
duplicate			$0.55^{7} \pm 0.009$
Inactivated by CySH + O_2			$0.55^{5} \pm 0.007$
duplicate			$0.56^{1} \pm 0.005$
Inactive on preparation	10	2	$0.78^{1} \pm 0.015$
Inactivated by CySH + O_2			$0.83^{5} \pm 0.026$
Inactivated by peroxide*	10	2	$0.13^{3} \pm 0.005$

* $1.0 \cdot 10^{-4}$ M activated gel-filtered papain, $2.0 \cdot 10^{-4}$ M sodium perborate (pH 7.4), 1.5 h at 25°.

of papain which had been inactivated by peroxide in the absence of cysteine was very slow in regaining activity.

Therefore it may be concluded that 'naturally' inactive papain and papain inactivated artificially in the presence of cysteine are identical.

The nature of the inactivation and activation reactions

The results in Fig. 1 indicate that inactivation of papain (PSH) by dimerization according to

$$2\ \mathrm{PSH} + \tfrac{1}{2}\ \mathrm{O_2} \rightarrow \mathrm{PSSP} + \mathrm{H_2O}$$

is either absent or very slow (*cf.* ref. 11). The conditions of Fig. 2 suggest that the inactivation occurs in two steps: air oxidation, catalyzed by heavy metals, of cysteine to cystine (Reaction 1) and inactivation of papain by disulphide exchange with cystine (Reaction 2):

$$2\ \mathrm{CySH} + \tfrac{1}{2}\ \mathrm{O_2} \rightarrow \mathrm{CySSCy} + \mathrm{H_2O} \qquad (1)$$

$$\mathrm{PSH} + \mathrm{CySSCy} \rightleftarrows \mathrm{PSSCy} + \mathrm{Cys} \qquad (2)$$

Of course, a more direct inactivation, according to

$$\mathrm{PSH} + \mathrm{CySH} + \tfrac{1}{2}\ \mathrm{O_2} \rightarrow \mathrm{PSSCy} + \mathrm{H_2O} \qquad (3)$$

is also possible. Activation would be Reaction 2 in the reverse direction. Inactivation predominates in the presence of cystine and of oxygen which constantly reoxidizes cysteine produced by the exchange reaction 2 until completion of the inactivation. Activation occurs when the solution containing papain *plus* cystine is 100-fold diluted (as for assay) and a large excess of cysteine or other suitable reducing agent is added.

The occurrence of Reaction 1 was established by amperometric titration of thiol groups. When inactivation was complete, thiol groups were absent from the reaction mixture. The occurrence of the mixed disulphide PSSCy was established by experiments with labelled cystine. Reactions 2 and possibly 3 require that a half-cystine residue is bound to inactive papain and removed again on activation. Samples of papain were inactivated in the presence of [^{35}S]cystine and subjected to gel filtration on a Sephadex G-25 column.

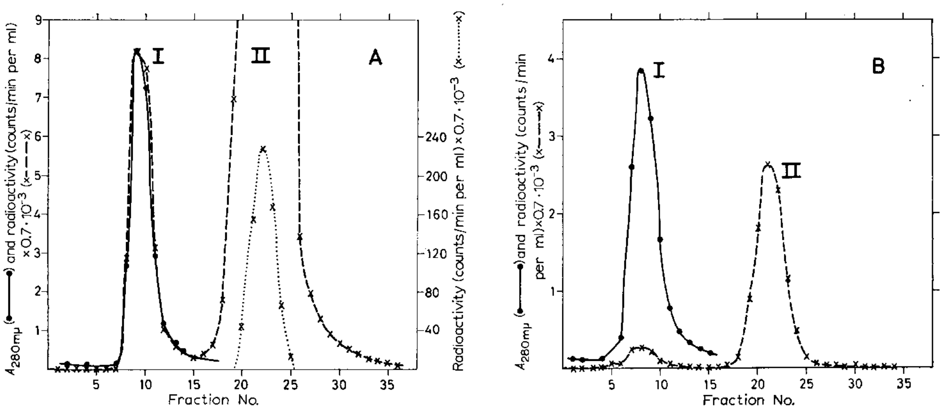

Fig. 3. Gel filtration of labelled papain. A, papain inactivated in the presence of labelled cysteine. B, the two main fractions of Peak I of A, activated with $5.0 \cdot 10^{-3}$ M (non-labelled) cysteine.

Fig. 3A shows an example of such an experiment. The ultraviolet light-absorbing papain peak, Peak I, manifestly carries radioactivity. Peak II contains the excess cystine. The two main papain-containing fractions of Peak I were combined. The papain was activated by making the solution 5 mM in (non-labelled) cysteine and was rechromatographed on a Sephadex column. Fig. 3B shows that, according to expectation, most of the radioactivity is removed from the papain and is eluted in a separate peak which corresponds with Peak II of Fig. 3A.

The low residual radioactivity like that of Peak I of Fig. 4B varied between 7 and 20% of the total protein-bound radioactivity in many experiments, being mostly around 10%. It is probably due to some non-specific side-reaction with the protein, since such low and irreversibly bound radioactivity was also observed if the 'inactivation' mixture contained papain without available thiol groups (after treatment with iodoacetic acid), or gelatin, and was absent if the 'inactivation' mixture contained no protein.

One would expect a 1:1 ratio of labelled half-cystine content and thiol content of the protein. This was indeed found. In 12 experiments like that in Fig. 3, after correction for the residual activity (Fig. 3B), a ratio of 0.98±0.15 was found. Using Sephadex columns a few weeks old, low ratios of 0.4–0.6 were sometimes observed. This corresponds with some trailing of the radioactivity after the ultraviolet absorption of the papain peak, indicating a slow removal of the half-cystine residue during the chromatographic run. This could be prevented by removing possible reducing material from the column by a previous treatment with KI_3 as described in the experimental section.

It may be concluded from the present results that inactive papain, prepared according to the procedure of KIMMEL AND SMITH, is a mixed disulphide with cysteine: PSSCy. Activation consists of removal of the half-cystine residue and concomitant liberation of the essential thiol group.

The effect of EDTA and cysteine on heavy-metal inhibition

In order better to define the function of EDTA and cysteine in preventing heavy-metal inhibition*, two types of experiments were conducted. In the first type heavy metal ions were added to papain in the presence of cysteine, in the absence of EDTA. In the second type EDTA was present and cysteine was absent.

For the former type of experiment, papain was (incompletely) activated solely with cysteine and assayed for esterase action in a pH-stat. After the run had progressed for a few minutes the desired amount of metal ions, dissolved in water, was added to the reaction mixture. The slopes of the progress curves just previously to and after the addition of metal ions were measured. The latter slope was corrected for the dilution caused by the addition of the metal ion solution and divided by the former slope. This ratio provides the percentage of activity remaining after the addition of the metal ions. The results are presented in Table II. Since there was always an excess of thiol concentration over heavy metal ion (M) concentration, the following equilibrium was most likely to predominate:

$$\mathrm{PSH} + \mathrm{M(SCy)_2} \rightleftharpoons \mathrm{PSMSCy} + \mathrm{CySH}$$

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* This effect is not to be confused with catalysis of oxidation as considered in the previous section. The catalytic oxidation is a slow process; the inhibition occurs instantaneously.

TABLE II

EFFECT OF DIVALENT METAL IONS ON THE ACTIVITY IN THE PRESENCE OF CYSTEINE, IN THE ABSENCE OF EDTA

Activity is presented as percentage of the activity in the absence of metal ions.

Divalent metal ion (mM)	*Cysteine (mM)*	*% activity*	*K*
Fe^{2+} 1.02	4.8	89	0.3
Pb^{2+} 1.03	4.8	68	1.3
Cu^{2+} 1.00	4.8	55	2.3
Hg^{2+} 1.02	4.7	42	3.7
Hg^{2+} 2.04	4.7	3	approx. 10
Cd^{2+} 0.051	4.8	43	120
Cd^{2+} 0.102	4.8	31	100
Cd^{2+} 0.51	4.8	15	43
Zn^{2+} 0.002	4.8	58	1750
Zn^{2+} 0.010	4.8	19	2000
Zn^{2+} 0.102	4.8	1.5	approx. 3000

The equilibrium constants $K = [\text{PSMSCy}]\,[\text{CySH}]/([\text{PSH}]\,[\text{M(SCy)}_2]$ were calculated and are included in Table II. Although K does not always seem to be quite independent of metal ion concentration, the results may serve as a guide and show that the bivalent ions Fe^{2+}, Pb^{2+}, Cu^{2+} and Hg^{2+} have no, or no great, preference for combining with papain as against cysteine. On the other hand, Cd^{2+} has about a 100-fold stronger affinity for papain than for cysteine. For Zn^{2+} this ratio is more than 1000. Apparently the effects of metal ions differ widely.

For the experiments in the absence of cysteine, papain was activated in the ordinary fashion and separated from EDTA and cysteine by gel filtration. $ZnCl_2$ or $HgCl_2$ was added to the combined papain-containing eluate fractions in quantities equimolecular to the papain concentration as determined by ultraviolet absorption. The activities of these preparations were tested in the presence of EDTA only, and in the presence of both EDTA and cysteine. The ratio of these activities provided the data of Table III.

It is remarkable that Zn^{2+} which, according to Table II, has such a stronger affinity for papain than for cysteine, is easily removed by EDTA, while Hg^{2+} is not. Thus EDTA but not cysteine is able to prevent inhibition by Zn^{2+}; cysteine but not EDTA is able to prevent inhibition by Hg^{2+}. This clearly demonstrates that neither

TABLE III

REMOVAL OF DIVALENT METAL IONS FROM PAPAIN BY EDTA IN THE ABSENCE OF CYSTEINE

Activity is presented as percentage of the activity in the presence of both EDTA and cysteine (5 mM). Concentration of metal ions is equal to concentration of papain, *i.e.* 0.7 M.

EDTA (mM)	*Metal ion*	*% activity*
1.0	Zn^{2+}	$\geqslant 90$
1.0	Hg^{2+}	3
5.0	Hg^{2+}	8.5

EDTA nor cysteine alone is capable of preventing all heavy-metal inhibition but that they supplement each other.

DISCUSSION

GLAZER AND SMITH[11] recently considered the possibility that the mixed disulphide of papain and cysteine, PSSCy, is the inactive form of papain after its preparation. They were able to show that performic oxidation of exhaustively dialyzed papain liberated small amounts of cysteic acid. Their results confirm our own in a qualitative way; apparently not, however, quantitatively, since three samples of papain gave only 0.04, 0.07 and 0.28 mole of free cysteic acid per mole of papain. Therefore GLAZER AND SMITH considered the mixed disulphide to be of little importance. On the other hand, their highest value of 0.28 mole of cysteic acid is, in any case, about half the value to be expected from papain samples like our own. The low recoveries obtained by these authors may possibly be due to the dialysis of their preparations, because according to our experience preparations of non-activated papain which had been subjected to dialysis could sometimes be activated only to a much lesser extent than the same papain preparations previous to dialysis.

SANNER AND PIHL[5] established the occurrence of Reaction 2 with another disulphide, *viz.* cystamine. The latter compound is capable of inhibiting papain (confirmed by the present author) and is bound to the enzyme as judged from experiments with labelled cystamine.

The occurrence of PSSCy as the inactive form of papain may of course be limited to preparations prepared according to KIMMEL AND SMITH. For instance, papain inactivated by perborate (Table I) must be subject to some other mechanism of activation. Furthermore, in the latex, papain may be present as a mixed disulphide with some unknown thiol compound. FERDINAND, STEIN AND MOORE[12] report a case similar to the present one: from a streptococcal protease an unknown (volatile) mercaptan was liberated on activation.

It is now apparent that the activation is not due to splitting of an intra-chain disulphide bond. Hence it is not surprising that no profound change of conformation occurs on activation as judged from optical rotatory dispersion measurements[10].

In view of the equilibrium of Reaction 2 and the possibility of oxidation, one should be cautious in using extremely low activator concentrations for kinetic experiments. Furthermore, the thiol compound used for the activation should be completely in the reduced state, otherwise the activation may be incomplete even at high activator concentration. This may be the reason why somewhat incomplete activation with BAL and with penicillamine has been reported (Table III of ref. 5), whereas other authors[3,6] found complete activation with BAL.

It must be pointed out that the reason why different preparations of papain, after activation, have different activities and different thiol contents, still remains unexplained. GLAZER AND SMITH[11] proposed that higher, non-reducible oxidation states of the thiol group may be involved. Since H_2O_2 is known to be produced during oxidation of thiol compounds by oxygen[13], and since a decrease of up to 50% of the optimum activity has sometimes been observed during inactivation in dilute cysteine (see above, *The conditions of reversible inactivation*) such an explanation seems likely but needs confirmation.

ACKNOWLEDGEMENT

The skilful technical assistance of Mrs. W. M. RUTGES-VAN DEN BREMER is gratefully acknowledged.

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