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EFFECT OF *p*H ON INHIBITION OF PAPAIN BY ZINC, CADMIUM AND MERCURIC IONS

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Summary

The effect of *p*H on the inhibitory actions of Hg, Zn and Cd has been investigated. Papain inhibited with Zn or Cd, in the presence of cysteine, has an optimum *p*H of 8.0 while the uninhibited papain and its Hg-derivative both have *p*H of 6.0 as their optimum *p*H, in phosphate buffer at 37°C. The maximum inhibitory effect of Hg was observed at *p*H 4.5. Zn, on the other hand, was observed to exert its maximum effect at *p*H 6.0 and Cd at *p*H 6.5. Both Zn and Cd derivatives were found to be more stable at high *p*H than the original papain and its Hg-derivative. The findings appear to suggest different binding sites for the metals studied.

Introduction

Papain, a sulphydryl enzyme has been known for a long time to interact with heavy metals (Krebs, 1930). Krebs showed that heavy metals were potent inhibitors of papain. Kimmel & Smith (1957), taking advantage of the interaction of Hg with the -SH group in papain, prepared the mercurial derivative of the enzyme which they were able to store over a very long time without much loss in the enzymic activity.

Esters and, presumably, peptides are hydrolysed through an acyl-enzyme pathway by papain with Cys-25 on the polypeptide chain of the enzyme acylated (Hinkle & Kirsch, 1971; Lowe & Williams, 1965; Stockell & Smith, 1957). A plot of the ratio of the catalysis constant (k_{cat}) and the Michaelis constant (Km) against *p*H follows a bell-shaped curve with optimal activity at about *p*H 6.0. This is caused by the ionisation of His-159 and Cys-25 with *pka* values of 4.2 and 8.2. The *p*H dependence of k_{cat} for deacylation follows the ionisation of a base of *pka* value of about 4.0. This may be attributed to His-159 since cysteine is blocked in the acyl enzyme (Fersht, 1977).

From the above analysis it is obvious that *p*H has a great influence on the activity of papain. The interaction of metals with papain has been found to be reversible (Agyeman, 1983; Sluyterman, 1974). The behaviour of the enzyme after the removal of the metal/inhibitor appears to suggest that the interaction does not cause any permanent damage to the enzyme (Agyeman, 1983). It is, however, possible that the behaviour of the enzyme with the metal still bound to it may be different. For instance the *p*H stabilities of the metal derivatives may not be the same as the original enzyme.

Enzymes are generally only active over a limited range of *p*H and, in most cases, a definite optimum *p*H is observed. Since enzymes are proteins containing many ionizable groups, they exist in a whole series of states of ionisation and the distribution of the total enzyme among the various ionic forms depend on the *p*H and the ionisation constants of various groups.

However, as the catalytic activity is usually confined to a relatively small *p*H range, it seems likely that only one of the ionic forms of the enzyme is catalytically active in such cases as suggested by Michaelis & Davidsohn (1911).

There is some evidence that the ionisation of groups in the protein which are remote from the active centre has little or no effect, while the ionic state of groups in or close to the active centre has a very large effect (Dixon & Webb, 1967). Since the binding of metals to the protein molecule is likely to modify the ionisation state of the molecule and, probably, its stability and optimal *p*H an attempt has been made to study the

effect of *p*H on the metal inhibition of papain.

Experimental

The papain used in this work had first been partially purified on an organo-mercurial agarose adsorbent, i.e. if affinity chromatography and then subjected to chromato-focusing (CF). Analysis of the CF-purified papain on isoelectric electrophoretic gel showed only a single band, indicating that the enzyme used was of a very high purity (Agyeman, 1983).

The study was carried using 1.0×10^{-3} *M* α-benzyol -L- arginine p-nitroanilide (BAPNA) dissolved in 50×10^{-3} M phosphate buffer covering *p*H ranges 4.5 to 9.0. The assaying of the enzyme was carried out using the amidase activity method (Arnon, 1965).

The enzyme was activated with 5.0×10^{-3} *M* cysteine and the *p*H of the enzyme fraction adjusted to the specified *p*H. The temperature of the reactants was maintained at 37°C. At time zero, the enzyme and the metal (Mx_2) at a known concentration which would not inhinbit the enzyme completely were added to the substrate also maintained at the same *p*H and temperature as the enzyme. The assay then continued at 37°C for 10 min. The reaction was terminated by the addition of 30% (V/V) acetic acid and the change in absorbance at 41nm (A_{410}) was measured using a Pye Unicam SP1700 Uv-Visible Spectrophotometer.

Results and discussion

The *p*H profiles obtained (Table 1, Fig. 1-3) show that the optimal *p*H for the hydrolysis of α -benzyol-l-arginine p-nitroanilide (BAPNA) in 50×10^{-3}*M* phosphate buffer by chromatofocusing (CF) purified papain was *p*H 6.0. The *p*H optimum for the mercurial derivative papain or Hg-inhibited enzyme *p*H 6.0, while for both the Zn- and Cd-inhibited enzyme it was *p*H 8.0. The Zn-inhibited papain showed a sub-optimum at *p*H 5.0 which appears to suggest at least two binding sites for the zinc. Both the uninhibited and the Hg-inhibited enzyme revealed a shoulder at

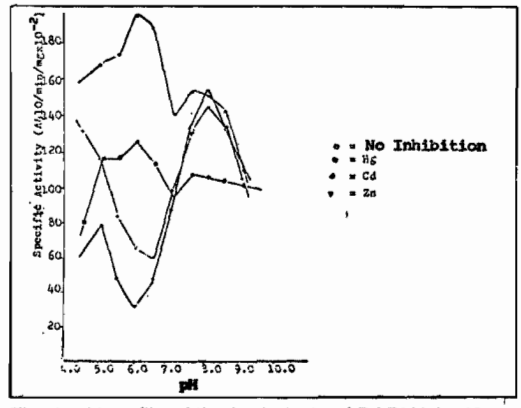

Fig. 1. *p*H profile of the hydrolysis of BAPNA in 50 *mM* phosphate buffer by CF-purified papain and its metal derivatives at 37 °C.

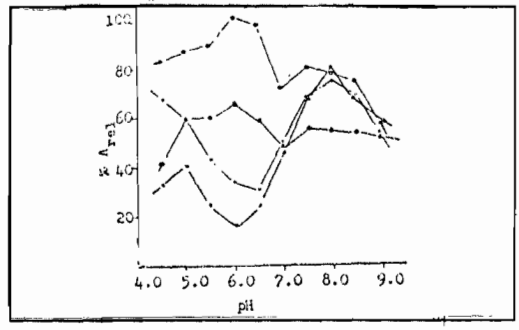

Fig. 2. Percent relative activity of the hydrolysis of BAPNA by CF-purified papain at different *p*Hs, at 37 °C (phosphate buffer) with respect to activity at optimal *p*H of the uninhibited enzyme (i.e. *p*H 6.0)

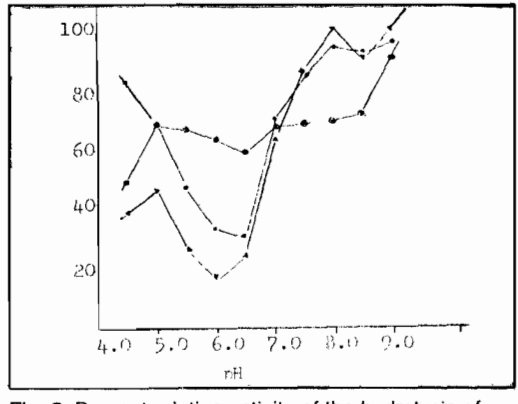

Fig. 3. Percent relative activity of the hydrolysis of BAPNA by CF-purified papain at different *p*Hs, at 37 °C (phosphate buffer), in relation to activity of the uninhibited enzyme at that *p*H.

TABLE I

Effect of pH *on CF-purified papain and its metal derivatives in the presence of cysteine*

(a) CF-purified papain (protein content = 0.016 mg/ml)

pH	*Specific activity (A410/min/mg protein)*	*Relative activity in relation to* pH *6.0 (%)*
4.5	$(160.00 \pm 3.20) \times 10^{-2}$	82.47 ± 2.00
5.0	$(168.00 \pm 7.13) \times 10^{-2}$	88.60 ± 4.24
5.5	$(172.00 \pm 5.23) \times 10^{-2}$	88.66 ± 3.04
6.0	$(194.00 \pm 7.52) \times 10^{-2}$	100.00 ± 3.04
6.5	$(188.00 \pm 6.31) \times 10^{-2}$	96.91 ± 3.36
7.0	$(138.00 \pm 3.72) \times 10^{-2}$	71.13 ± 2.70
7.5	$(153.00 \pm 4.31) \times 10^{-2}$	78.87 ± 2.82
8.0	$(150.00 \pm 2.73) \times 10^{-2}$	77.32 ± 1.82
8.5	$(141.00 \pm 4.22) \times 10^{-2}$	72.68 ± 2.99
9.0	$(109.00 \pm 2.33) \times 10^{-2}$	56.19 ± 2.14

(b) Papain inhibited with $ZnCl_2$

pH	*Specific activity*	*Relative activity A *(%)*	*Relative activity B *(%)*
4.5	$(62.50 \pm 1.31) \times 10^{-2}$	32.22 ± 2.10	39.06
5.0	$(78.13 \pm 1.72) \times 10^{-2}$	40.27 ± 2.26	46.51
5.5	$(46.88 \pm 0.82) \times 10^{-2}$	24.17 ± 1.75	27.26
6.0	$(31.25 \pm 0.51) \times 10^{-2}$	16.11 ± 1.63	16.11
6.5	$(46.88 \pm 1.00) \times 10^{-2}$	24.17 ± 2.13	24.94
7.0	$(87.50 \pm 1.41) \times 10^{-2}$	45.10 ± 1.61	62.41
7.5	$(132.81 \pm 2.11) \times 10^{-2}$	68.46 ± 1.59	86.80
8.0	$(153.13 \pm 2.72) \times 10^{-2}$	78.93 ± 1.78	102.19
8.5	$(128.13 \pm 2.12) \times 10^{-2}$	66.05 ± 1.66	90.87
9.0	$(110.94 \pm 1.83) \times 10^{-2}$	57.19 ± 1.65	101.78

Relative activity A* = Relative activity in relation to the activity of the uninhibited enzyme at the optimum *p*H.

(c) Papain inhibited with $CdCl_2$

pH	*Specific activity*	*Relative act. A*(%)*	*Rel. act. B* (%)*
4.5	$(131.25 \pm 2.00) \times 10^{-2}$	67.66 ± 1.52	82.03
5.0	$(115.63 \pm 1.75) \times 10^{-2}$	59.60 ± 1.51	68.83
5.5	$(82.51 \pm 0.92) \times 10^{-2}$	42.53 ± 1.12	47.97
6.0	$(65.63 \pm 0.71) \times 10^{-2}$	33.83 ± 1.08	33.83
6.5	$(59.38 \pm 0.53) \times 10^{-2}$	30.61 ± 0.89	31.59
7.0	$(96.88 \pm 1.51) \times 10^{-2}$	49.94 ± 1.56	70.20
7.5	$(129.69 \pm 1.32) \times 10^{-2}$	66.85 ± 1.02	84.77
8.0	$(143.75 \pm 2.11) \times 10^{-2}$	74.10 ± 1.47	95.83
8.5	$(131.25 \pm 1.35) \times 10^{-2}$	67.66 ± 1.03	93.09
9.0	$(103.13 \pm 2.21) \times 10^{-2}$	53.16 ± 2.14	94.62

(d) Papain inhibited with $HgCl_2$

pH	*Specific activity*	*Rel. act. A*(%)*	*Rel. act. B* (%)*
4.5	$(79.69 \pm 0.48) \times 10^{-2}$	41.08 ± 0.6	49.81
5.0	$(115.63 \pm 1.12) \times 10^{-2}$	59.60 ± 0.97	68.83
5.5	$(115.63 \pm 2.13) \times 10^{-2}$	59.60 ± 1.84	67.23
6.0	$(125.00 \pm 4.85) \times 10^{-2}$	64.43 ± 3.88	64.43
6.5	$(112.50 \pm 2.53) \times 10^{-2}$	57.99 ± 2.25	59.84
7.0	$(93.75 \pm 1.52) \times 10^{-2}$	48.33 ± 1.62	67.94
7.5	$(106.25 \pm 1.83) \times 10^{-2}$	54.77 ± 1.72	69.44
8.0	$(104.63 \pm 1.42) \times 10^{-2}$	53.93 ± 1.36	69.75
8.5	$(103.00 \pm 4.56) \times 10^{-2}$	53.09 ± 4.43	73.05
9.0	$(100.25 \pm 2.81) \times 10^{-2}$	51.55 ± 2.80	91.97

Relative activity B* = Relative activity in relation to the activity of the uninhibited enzyme at the optimum *p*H.

*p*H 7.0 - 8.0.

The results also show the effect of *p*H on the sequestening effect of cysteine as well as the inhibitory effects of the metals. Hg appears more inhibiting at low *p*H (*p*H 4.5) than at high *p*H. This is not entirely surprising since Sluyterman (1966) has reported that the active centre of papain contains a group of *pka* 4 which affects substrate binding. This may also suggest that cysteine may not be a very effective sequestening agent at low *p*H. The sequestening effects may be effective at high *p*Hs and, infact, cysteine has been found to activate papain at *p*H 6.0-6.5 (Agyeman, 1983; Sluyterman, 1974).

It was observed that *p*H 4.5 - 8.0 have relatively little effect on Hg inhibition below *p*H 8.5. Zinc was found to be most effective at *p*H 6.0 but ineffective at *p*H 5.0 and *p*H < 7. 0. Cadmium, on the other hand, was found to be most effective at *p*H 6.5 but ineffective at *p*H < 5.0 and >7.0. This may suggest more than one binding sites for Zn and Cd with inhibitory effects. The decreasing inhibitory effects of the metals may be due to the fact that with increasing *p*H the positive charges on the enzyme molecule decrease (i.e, $N^+ \rightarrow N$), resulting in the competition between an increasing variety of groups for the metals. This reduces the localized inhibitory effect and the enzyme becomes more effective despite the fact that the inhibitor is still present.

Moreover, papain is believed to be composed of a single polypeptide chain of 212 amino acids. Kinetic studies have shown that the active site, Cys-25, can accommodate seven amino acids, four on the acyl side, S_4 - S_1 and three on the amino side, S_1 - S_3 (Mitchell, Chaiken & Smith, 1970). Esters and, presumably, peptides are hydrolysed through an acyl-enzyme pathway with Cys-25 acetylated (Hinkle & Kirsh, 1971; Lowe & Williams, 1965; Stockell & Smith, 1957).

A plot of k_{cat}/K_m against *p*H follows a bell-shaped curve with optimal activity at about *p*H 6.0. This is caused by the ionisation of His-159 and Cys-25 with *pka* values of 4.2 and 8.2 respectively. The *p*H dependence of k_{cat} for deacylation follows the ionisation of a base of *pka* of about 4. This may be attributed to His-159 since cysteine is blocked in the acyl-enzyme (Fersht, 1977).

From the above account it appears obvious that the catalysis of papain depends very much upon the dissociation of Cys-25 to enable a tetrahedral intermediate, necessary for the catalysis, to be formed (Fig. 4). It appears that if His-159 is bound by any inhibitor the whole catalytic process may be inhibited unless self-dissociation of the Cys-25 is promoted by increase in environmental *p*H. It must be realised that the acceptance of proton from Cys-25 by His-159 is only possible at *p*H values at which the His-159 is not protonated and at values at which -SH is not exceedingly dissociated (i.e. *p*H > 8.0).

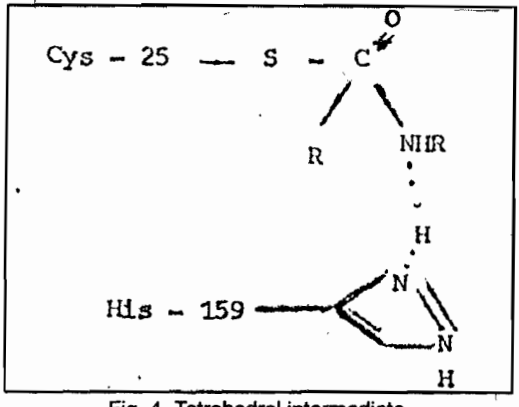

Fig. 4. Tetrahedral intermediate

On the basis of the above assertion it can be inferred that the shift in the optimal *p*H from *p*H 6.0 to 8.0 on the presence of Zn and Cd may indicate probably that one of the binding sites of Zn or Cd is His-159. Since at *p*H > 8.0 self dissociation of Cys-25 may be promoted and the participation of His-159 may then not be very important. It must, however, be conceeded that several other factors may account for this shift. For instance, if these metals bind at sites close to the active sites of the enzyme it may affect the ionic state of the enzyme and, hence, cause a shift in the optimum *p*H.

The self-dissociation of Cys-25 at high *p*H may

also account for the shoulder observed in the *p*H profiles of the uninhibited and Hg-inhibited papain. High *p*H causes denaturation of protein due, among other factors, to the breaking of hydrogen bonds. This would lead to the unfolding of the protein molecule. The self-dissociation of the Cys-25 would tend to increase the enzymic hydrolysis of the substrate, but because of the unfolding of the molecule, there might be a lengthening of the distance between the active site and the related sites, e.g. His-159, thus considerably reducing any enzymic activity this self-dissociation of Cys-25 would have achieved.

The effect of *p*H on enzymic activity is so complex that it is not easy to assign one particular reason to the results obtained. However, from the results it appears that the *p*H effects, together with other studies like the behaviour of the inhibited papain on chromatographic adsorbents such as organomercurial agarose, sephacryl-S 300, zinc chelate, arginine-sepharose 4B, etc., may help elucidate the binding sites of these metals (Agyeman, 1983).

The conclusion one can draw from this work is that both Zn and Cd-inhibited papain appear to be more stable at high *p*H than the uninhibited papain or its mercurial derivative, and that His-159 appears a possible binding site for Zn or Cd. Hg may bind to Cys-25 as reported by other workers like Sluyterman (1974), Wolthers & Kalk (1970) and Kimmel & Smith (1957).

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References

AGYEMAN, K. O. G. (1983) *The interaction of metals with plant proteases of the thiol type* (Ph.D. Thesis). Procter Department of Food Science, University of Leeds, U.K.

ARNON, R. (1965) The reaction of papain with antipapain. *Immunochemistry*, **2**, 107-114.

DIXON, M. & WEBB, E. C. (1967) *Enzymes*, 3rd ed. London: Longman Group Ltd.

FERSHT, A. (1977) *Enzyme structure and mechanism.* Reading: W. H. Freeman and Co. Ltd.

HINKLE, P. M. & KIRSCH, J. F. (1971) *Biochemistry* **10**, 2717.

KIMMEL, J. R. & SMITH, E. L. (1957) The properties of papain. *Adv. Enzymol.* **19**, 267-334.

KREBS, H. A. (1930) Versuche über die proteolytische wirkung des papain. *Biochem. Z.*, **220**, 289-303.

LOWE, G. & WILLIAMS, A. (1965) *Biochem.* J. **35**, 189-193.

MICHAELIS, L. & DAVIDSOHN, B. (1911) Die Wirkung der Wasser stoffionen auf des Invertin. *Biochem. Z.* **35**, 386-412.

MITCHELL, R. E. J., CHAIKEN, I. M. & SMITH, E. L. (1970) The complete amino acid sequence of papain-additions and compressions. *J. Biol. Chem.* **245**, 3485-3492.

SLUYTERMAN, L. A. A. E. (1974) Product inhibition of papain action. *Biochim. Biophys. Acta* **85**, 316-312.

SLUYTERMAN, L. A. A. E. (1966) Substrate binding by non-activated papain. *Biochim. Biophys. Acta* **133**, 577-586.

STOCKELL, A. & SMITH, E. L. (1957) Kinetics of papain action. 1. Hydrolysis of Benzo/L-L-Agininamide. *J. Biol. Chem.* **227**, 1-26.

WOLLTHERS, B. G. & KALK, G. H. (1970) Binding of competitive (product) inhibitors to papain in the active and mature state. *Biochim. Biophys. Acta* **198**, 556.

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