INHIBITION OF -SH ENZYMES BY AN IMPURITY IN COMMERCIAL SAMPLES OF DIISOPROPYLPHOSPHOFLUORIDATE*

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The inhibition of animal proteases and esterases by diisopropylphosphofluoridate (DFP) is well documented (Cohen et al., 1959), but the inhibition of -SH containing plant proteases is equivocal. For example, Jansen et al. (1948) and Kimmel and Smith (1954) reported DFP not to inhibit papain, whereas Masuda (1959) and Heinicke and Mori (1959) found an inhibitory effect; inhibition of bromelain was reported by Heinicke and Mori (1959), Ota et al. (1962), and Ebata et al. (1962), but not found by Murachi and Neurath (1959). Ebata and Yasunobu (1963) have described the crystallization of an inactive diisopropylphosphoryl derivative of chymopapain, although Murachi (1963) states that DFP reacts with -SH enzymes without affecting their enzymic activity. It is hoped that the observations recorded in this preliminary communication will serve to reconcile these conflicting reports.

Ficin and crystalline papain, both -SH containing plant proteases, were prepared by the methods of Hammond and Gutfreund (1959) and Kimmel and Smith (1954) respectively. Chymotrypsin, a 2x crystallized preparation, was purchased from Worthington Biochemical Corporation, Freehold, N.J. The activity of these enzymes was measured by the method of Kunitz (1947). Two samples of DFP were purchased, one from the Aldrich Chemical Co., Milwaukee, Wisconsin, and the other from Merck and Co., Rahway, N.J.

The data in Fig. 1 show that the sample of Aldrich DFP inhibited both

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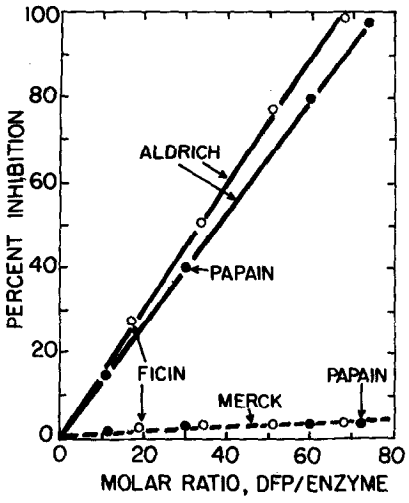

Figure 1. Inhibition of ficin and papain by Aldrich and Merck DFP. In 1 ml: 0.1 M borate buffer, pH 8, 0.1 M KCN, 0.1 μmole enzyme. Add 1.5 ml. isopropanol with DFP as indicated. Assay enzyme after 30 min. at 30°.

Table I.

Effect of histidine-Cu hydrolysis of Aldrich DFP on ficin and chymotrypsin activity.

Hydrolysis	Inhibition	
	Ficin	Chymotrypsin
Min. at 30°	%	%
0	54	100
2	54	86
30	45	13
60	43	7

In 8 ml.: 25 μg DFP plus 625 μ moles each of histidine and $CuSO_4$, held at pH 7.6 in pH-stat. Assay enzyme after 30 min. at 30°. % inhibition calculated from controls without DFP.

ficin and papain to a similar degree, whereas the Merck DFP was not inhibitory. Chymotrypsin was inhibited to the same extent by both DFP samples. In agreement with the reports by Masuda (1959) and Ebata et al. (1962), 0.05 M cysteine protected ficin and papain from DFP inactivation.

To test whether DFP per se was responsible for enzyme inhibition by the Aldrich preparation, the latter was hydrolyzed catalytically with histidine and $CuSO_4$ (Wagner-Jauregg, et al., 1955) and tested periodically for inhibitory activity towards ficin and chymotrypsin. As the data in Table I show,

the inhibition of chymotrypsin decreased with time whereas ficin was essentially unaffected.

When Aldrich DFP (2 gm.) was distilled under reduced pressure, most of the starting material was recovered in fraction I (25°-36°, 160 microns Hg) which was trapped in a receiver immersed in dry ice and acetone. Two other fractions, II and III, were collected in 4 ml. isopropanol at 37°-46° and 47°-56° respectively. The residue, fraction IV, was dissolved in 1 ml. of isopropanol. Table II shows that the inhibitor of ficin resides in fraction IV, although this fraction is still contaminated with DFP as evidenced by the inhibition of chymotrypsin.

Table II.

Fractional distillation of Aldrich DFP; effect of various fractions on activities of ficin and chymotrypsin

Fraction	Inhibition	
	Ficin	Chymotrypsin
	%	%
I	0	100
II	0	100
III	0	100
IV	95	82

In 1 ml.: 0.1 M borate buffer, pH 8, 0.005 μ mole enzyme, 10 μl of test fraction. Assay enzyme after 60 min. at 37°.

It was of interest to ascertain whether the inactivation observed here involved the stoichiometric incorporation of phosphorus as claimed for papain (Masuda, 1959) and chymopapain (Ebata and Yasunobu, 1963). Ficin, treated with distilled DFP (fraction I) or the purified inhibitor (fraction IV), was precipitated with 0.5 saturated $(NH_4)_2SO_4$, dialyzed until salt free, and analyzed for P (Bartlett, 1959). Ficin inactivated by fraction IV contained 0.25 mole P/mole whereas the uninhibited enzyme treated with fraction I had 1.13 mole P/mole. It is evident that inactivation and phosphorus incorporation are unrelated manifestations of two independent reactions.

Table III.

Effect of Aldrich DFP and purified inhibitor (fraction IV) on activity and -SH groups of ficin.

DFP/enzyme	Inhibition	-SH/enzyme*
mole/mole	%	mole/mole
0	0	0.60
17	25	0.45
34	51	0.30
51	78	0.20
68	97	0.18
85	98	0.14
Fraction IV	92	0.15

Procedure as described in Fig. 1 and Table II.
* Determined by the method of Ellman (1959).

Table III shows that the inhibition of ficin by Aldrich DFP or fraction IV was accompanied by a loss in -SH groups. Preliminary chromatographic experiments have revealed the formation of an unidentified component when cysteine itself was treated with fraction IV. Work on the further purification and characterization of the inhibitor and on the nature of its reaction with -SH groups is in progress.

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