EFFECT OF DIISOPROPYLPHOSPHOROFLUORIDATE ON PAPAIN, CHYMOPAPAIN AND BROMELAIN[1]

Mitsuo Ebata[2], Joyce S. Tsunoda[3] and Kerry T. Yasunobu

Department of Biochemistry and Biophysics,

University of Hawaii, Honolulu, Hawaii

Received August 6, 1962

The inhibition of animal proteases and hydrolases by diisopropylphosphorofluoridate (DFP) has been studied in detail and the importance of this reaction in mechanistic studies has been adequately covered by others (Jansen et al. 1948, Cohen et al. 1959). However, the reports concerning DFP-inhibition of sulfhydryl proteases have been somewhat contradictory. For example, Jansen and co-workers (1948) and Kimmel and Smith (1954) found that untreated and cysteine-activated papain were not inhibited by DFP. Recently, Masuda (1959) showed that DFP inhibited cyanide-activated papain. Heinicke and Mori (1959) also reported on the DFP inhibition of bromelain, ficin and papain, although the results were variable. Ota et al. (1961) demonstrated that cyanide-activated bromelain was inhibited by DFP, while cysteine-activated bromelain was not inhibited appreciably. From these findings both Masuda and Ota concluded that sulfhdryl plant proteases are inhibited by DFP when cyanide is the activating agent but not when cysteine is the activating agent.

In this report, we wish to report that papain, chymopapain and bromelain are inhibited by DFP consistently. Some of the factors affecting DFP inhibition

---

1 Supported by grants from the National Science Foundation.

2 On leave from Osaka University, Osaka, Japan

3 National Science Foundation predoctoral fellow.

which were investigated includes: (1) The nature of the activating agent; (2) concentration of the activating agent; and (3) the source of DFP.

Twice-crystallized papain was prepared by the method of Kimmel and Smith (1954) and also by Masuda's method (1959). The specific activities of the preparations were 4 and 3, respectively. Twice-recrystallized chymopapain was prepared according to the method of Ebata and Yasunobu (1962) and showed a specific activity of 1.0. Bromelain (kindly supplied by Dr. R. Heinicke, Dole Pineapple Co., Honolulu, Hawaii) was prepared by the method of Murachi and Neurath (1959), and this preparation corresponded to fraction 5 of their preparation scheme. The bromelain preparation was homogeneous in the ultracentrifuge and exhibited a specific activity of 0.5. To 1 ml of papain containing 3 mg of protein (6.4 mg of chymopapain and 18.0 mg of bromelain) was added 1 ml of cysteine or sodium cyanide solution and the activation process was allowed to proceed for 60 minutes at 35°. The mixture was then diluted with 8-15 ml of 0.1 M phosphate buffer, pH 7.2. To 1 ml of this diluted enzyme solution, 1 ml of DFP was added and after 20 minutes incubation, activity measurements were determined using the method of Kunitz (1947).

Figs. 1, 2 and 3 show the effect of DFP on cysteine- and cyanide-activated papain, chymopapain and bromelain, respectively. All of these enzymes were readily inhibited by DFP after activation with cyanide. The results with papain and bromelain agree with the results of Masuda (1959) and Heinicke and Mori (1959), respectively. The DFP inhibition was found to be dependent upon cyanide concentration. The enzymes activated by cysteine were also consistently inhibited by DFP provided that the cysteine concentrations were low relative to the DFP concentrations. A remarkable inhibition was observed when the DFP was added to the cysteine-free chymopapain solution (Fig. 2, III). The cysteine-free enzyme was prepared by the use of a small column consisting first of 3 cm of Dowex 1 (OH^- form), 3 cm of Dowex 50 (H^+ form) and 12 cm of Sephadex G-50. The results obtained indicates that excess cyanide, and especially cysteine, prevents the reaction between DFP and enzyme. As pointed out by Masuda (1959), this is not

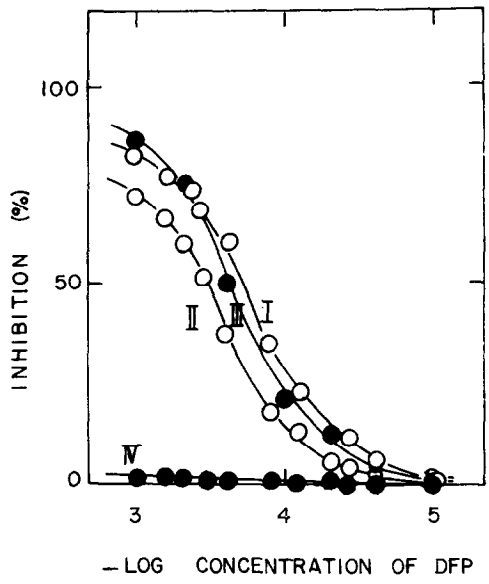

Fig. 1. Effect of DFP on cyanide- and cysteine-activated papain. 30 μg of enzyme prepared by the method of Kimmel and Smith was dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.2. The enzyme was activated with 0.1 M or 0.25 M sodium cyanide or 0.1 M cysteine for 60 minutes at 35° and then treated with Mann DFP. After 20 minutes incubation, the activity was analyzed as previously described (Ebata and Yasunobu, 1962).

I: Activated by 0.1 M NaCN (final CN^- concentration, 1.5×10^{-3} M).
II: Activated by 0.25 M NaCN (final CN^- concentration, 3.75×10^{-3} M).
III: Activated by 0.01 M cysteine (final cysteine concentration, 1.5×10^{-4} M).
IV: Activated by 0.1 M cysteine (final cysteine concentration, 1.5×10^{-3} M).

due to a reaction between DFP and enzyme, but appears to be due to the binding of the activating agent in the vicinity of the "active site".

It is suggested that the neglect of any of the following factors might account for the failure of some investigators to observe inhibition by DFP: (a) The importance of the nature of the activating agents as reported by Masuda (1959). (b) The importance of the concentration of the activating agent used. In cases where DFP-inhibition have not been observed, cysteine has been used as the activating agent; and as pointed out earlier, cysteine at proper concentrations prevents DFP inhibition of these enzymes. Preferably, the activating agents should be removed prior to DFP treatment. (c) The method of enzyme preparation may possibly be important. Some of the previous investigators

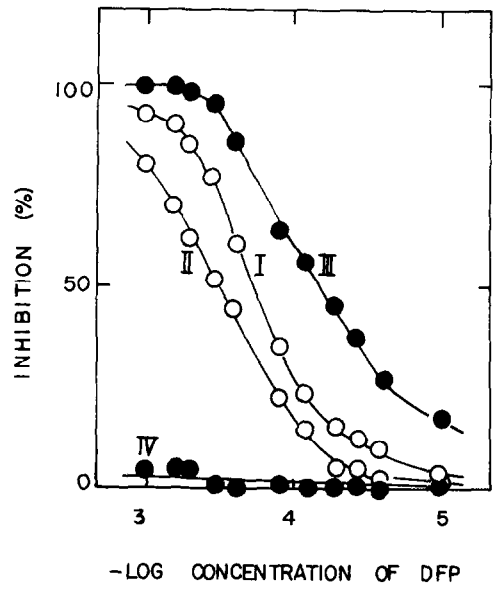

Fig. 2. Effect of DFP on cyanide- and cysteine-activated chymopapain. Analytical methods as described in Fig. 1. Enzyme concentration, 80 μg/ml.
I: Activated by 0.1 M NaCN (final CN^- concentration, 2.5×10^{-3} M).
II: Activated by 0.25 M NaCN (final CN^- concentration, 6.25×10^{-3} M).
III: Cysteine-free chymopapain activated by 0.1 M cysteine.
IV: Activated by 0.1 M cysteine (final cysteine concentration, 2.5×10^{-3} M).

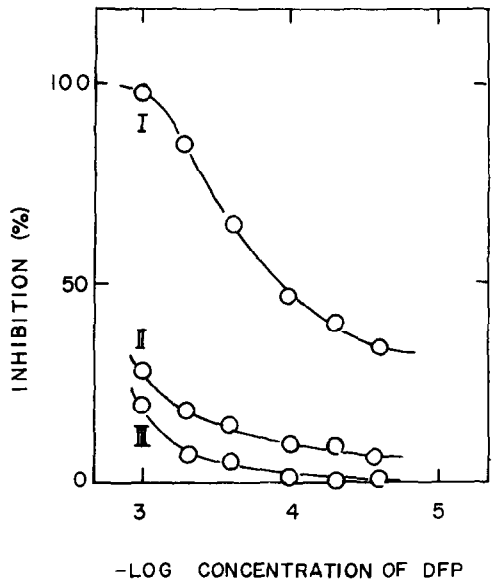

Fig. 3. Effect of DFP on cysteine-activated bromelain. Assay procedures were similar to those described under Fig. 1, except that bromelain concentrations were 132 μg/ml.
I: Activated by 0.01 M cysteine (final cysteine concentration, 1.5×10^{-4} M).
II: Activated by 0.05 M cysteine (final cysteine concentration, 7.4×10^{-4} M).
III: Activated by 0.1 M cysteine (final cysteine concentration, 1.5×10^{-3} M).

have used the activating agent, cysteine, at each step of the purification procedure. This may somehow prevent DFP from inhibiting these enzymes. However, as shown in Table I, the concentration of DFP required for 50% inhibition of papain prepared by Masuda's method (1959) and Kimmel and Smith's method (1954) was very similar. (d) The source of DFP was found to be very important (Table I). Aldrich DFP was much more effective than Mann DFP in inhibiting the enzymes. Mann DFP was reported to be a pure product.

Table I

DFP Concentration Required for 50% Inhibition of Papain and Chymopapain

Enzyme	Aldrich DFP M/L	Mann DFP M/L
Papain (prepared by the method of Kimmel and Smith)	5.2×10^{-5}	2.2×10^{-4}
Papain (prepared by the method of Masuda)	4.1×10^{-5}	2.0×10^{-4}
Chymopapain	1.1×10^{-5}	2.0×10^{-4}

Papain, 30 μg/ml, and chymopapain, 80 μg/ml, were dissolved in 0.1 M phosphate buffer, pH 7.2. The enzymes were activated by 0.1 M NaCN (final CN^- concentration was 2.5×10^{-3} M) and assayed as described in Fig. 1.

In summary, we have observed consistent inhibition of papain, chymopapain and bromelain by DFP, regardless of the compound used as the activating agent provided that the latter is removed prior to DFP-treatment. The DFP inhibition of these enzymes might possibly be related in some way or other to the mechanism of enzymatic action. Kimmel and Smith (1957) have proposed that a sulfhydryl group and a carboxyl group are the functional groups responsible for catalysis by these enzymes. Since the sulfhydryl proteases appear to be inhibited by DFP, serious consideration should be given to the possibility that serine is present in the active centers of these enzymes.

References

Cohen, J. A., Oosterban, R. A., Jansz, H. S., and Berends, F., J. Cell. and Comp. Physiol., 54, Suppl. 1, 231 (1959).
Ebata, M., and Yasunobu, K. T., J. Biol. Chem., 237, 1086 (1962).
Heinicke, R. M., and Mori, R., Science, 129, 1678 (1959).
Jansen, E. F., Nutting, M. D. F., and Balls, A. K., J. Biol. Chem., 175, 975 (1948).
Kimmel, J. R., and Smith, E. L., J. Biol. Chem., 207, 515 (1954).
Kimmel, J. R., and Smith, E. L., Adv. in Enzymology, 19, 267 (1957).
Kunitz, M., J. Gen. Physiol., 30, 291 (1947).
Masuda, T., J. Biochem., 46, 1569 (1959).
Murachi, T., and Neurath, H., J. Biol. Chem., 235, 99 (1960).
Ota, S., Fu, T. H., and Hirohata, R., J. Biochem., 49, 532 (1961).