Alkylphosphorylation of Stem Bromelain by Diisopropylphosphorofluoridate without Inhibition of Proteinase Activity*

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ABSTRACT: The effect of three different preparations of diisopropylphosphorofluoridate (DFP) on the caseinolytic activity of stem bromelain has been studied in the presence of either L-cysteine or KCN added as the activator. The results obtained, together with the data for titration of SH content of DFP-treated enzymes, indicate that DFP *per se* has no inhibitory effect on stem bromelain, while impurities associated with some DFP preparations may cause an inhibition by irreversibly attacking the essential sulfhydryl (SH) group of the enzyme. Gas chromatographic detection of impurities in DFP preparations has been attempted. When stem bromelain is incubated with more than 100-fold molar excess of DFP at pH 8.2 for 3 hr at 30°, approximately 1 mole of phosphorus is incorporated into 1 mole of the enzyme protein without inhibition. Such phosphorylation also occurs even when the essential SH group of the enzyme has been blocked by mercuric ion. The phosphorus incorporated is so firmly bound to the enzyme protein that it can neither be removed by gel filtration nor by repeated ammonium sulfate precipitation and dialysis, indicating a true alkylphosphorylation. The phosphorylation of stem bromelain by DFP is enhanced with an increase in pH above 8, reaching a ratio of 2.4 moles of phosphorus/mole of protein at pH 10.1. The phosphorylation proceeds rather slowly at pH 8.2, the rate and the extent of the reaction being dependent on the concentration of DFP even when the latter is present in more than 100-fold molar excess. These findings suggest that the mechanism and the site of alkylphosphorylation of stem bromelain may differ from those of chymotrypsin.

In an earlier communication by Murachi and Neurath (1960) it was reported that diisopropylphosphorofluoridate (DFP) did not inhibit hydrolytic activity of stem bromelain on casein. The finding was in accord with the results obtained by Jansen *et al.* (1948) and Kimmel and Smith (1954) that DFP had no inhibitory effect on papain. On the other hand DFP was found to inhibit strongly and irreversibly chymotrypsin and trypsin. The inhibition was also found to occur with other proteolytic enzymes like thrombin, elastase, and subtilisin (Hartley, 1960). The inhibition by DFP has such characteristic features common to all these proteinases that (1) DFP rapidly alkylphosphorylates the enzyme protein, (2) the introduction of a single diisopropylphosphoryl (DIP)[1] group per molecule of enzyme protein results in a total loss of activity, and (3) the site of alkylphosphorylation of the enzyme protein is always one of the serine hydroxyls as evidenced by isolation of either *O*-phosphorylserine from an acid hydrolysate of DIP enzyme (Schaffer *et al.*, 1953) or a peptide containing an *O*-DIP-seryl residue from an enzymatic hydrolysate (Cohen *et al.*, 1959). These facts have lent strong support to the view that DFP is a specific agent or a quasi-substrate (Koshland, 1960) which blocks the active center of these enzymes by alkylphosphorylation.

The present investigation was undertaken to reconfirm the results of our earlier experiment (Murachi and Neurath, 1960) that the bromelain-catalyzed hydrolysis of casein was not inhibited by DFP. The reconfirmation was necessary particularly in view of the results reported by other investigators that were at variance with ours. For example, Heinicke and Mori (1959) suggested that DFP is a specific inhibitor of SH proteinases; Ota *et al.* (1961) found an inhibitory effect on bromelain; Masuda (1959) and Ebata and Yasunobu (1963) isolated inactive, phosphorus-containing papain and chymopapain, respectively, after incubating the enzymes with DFP in the absence of cysteine. The conclusion we have drawn from the results of the earlier and the present experiments is that DFP *per se* has no inhibitory effect on stem bromelain, while impurities associated with some DFP preparations may cause an inhibition by irreversibly attacking the essential SH group of the enzyme. A similar conclusion has been arrived at more recently by Gould *et al.* (1963) and Gould and Liener (1965) from the experiments with ficin and papain. They have demonstrated that there exists in some commercial preparations of DFP an impurity which reacts with sulfhydryl (SH) groups of cysteine and enzyme proteins.

In the course of the present investigation we have also

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[1] The abbreviation used is: DIP, diisopropylphosphoryl.

found that, when stem bromelain was incubated with DFP at an alkaline pH, phosphorus was incorporated into the enzyme protein without inhibition of the proteinase activity. Similar phenomena were also encountered in cases of papain, Taka-amylase A, and bovine serum albumin (Murachi, 1963) and egg white lysozyme (Murachi and Inagami, 1963). Gould and Liener (1965) have likewise reported the phosphorylation of ficin by DFP. The present paper describes the details of experiments with stem bromelain. It is demonstrated that the phosphorylation by DFP of stem bromelain differs from that of chymotrypsin in many respects, suggesting a different mechanism and site of the reaction on the surface of the enzyme protein.

Materials and Methods

Stem Bromelain. The enzyme preparation used was isolated from crude "Bromelain" (lots 181 and 182) from the Hawaiian Pineapple Co., Honolulu, Hawaii,[2] by the method of Murachi *et al.* (1964). Fraction 6 was employed for the present experiment. The concentration of the enzyme protein was determined by measuring the absorbancy at 280 mμ with a Hitachi Model EPB-U spectrophotometer. $A^{1\%}_{1\,cm}$ of 19.0 and the corresponding molar absorbancy of 6.33×10^4 were used for calculating the concentration of native stem bromelain (Murachi *et al.*, 1964). The same factors were also employed for calculating approximate concentrations of DIP stem bromelain. Since DIP stem bromelain was later found to contain a DIP group at the tyrosine residues (Murachi and Inagami, 1963; Murachi *et al.*, 1965), the molar absorbancy at 280 mμ of a DIP enzyme must be less than that of the native enzyme. However, the difference was to be only 2% of 6.33×10^4, for example, with a specimen containing one DIP-tyrosyl residue per molecule, and hence no correction was made.

Other Enzyme Preparations. Crystalline papain was prepared from dried papaya latex, obtained from Nagase Co., Ltd., Osaka, Japan, according to the method of Kimmel and Smith (1954). $A^{1\%}_{1\,cm}$ at 278 mμ of 25.0 (Glazer and Smith, 1961) was used for calculating papain concentration. The molecular weight of papain was assumed to be 21,000 (Smith and Kimmel, 1960). *Crystalline ficin* was a product of Mann Research Laboratories, Inc., New York, N. Y. (lot C2094).[3] The concentration of ficin was determined by assuming $A^{1\%}_{1\,cm}$ at 280 mμ = 22.4 (Gould and Liener, 1965). *Trypsin* (lot 813–4) was a product of Worthington Biochemical Corp., Freehold, N. J. $A^{1\%}_{1\,cm}$ at 280 mμ of 14.4 (Davie and Neurath, 1955) was used for calculating trypsin concentration. The molecular weight of trypsin was assumed to be 24,000 (Cunningham, 1954). *Bacillus subtilis proteinase* was obtained from Nagase Co. $A^{1\%}_{1\,cm}$ at 280 mμ of 8.8 and a molecular weight of 30,500 were used for determining molar concentration of the proteinase (Hagihara, 1960).

Proteinase Activity. Hydrolysis of casein by stem bromelain was carried out at pH 7.2 and at 30° according to the procedure described in an earlier communication (Murachi *et al.*, 1964). Proteinase activities of other enzymes were also assayed in the same manner using appropriate amounts of the enzyme protein per incubation tube. In the cases of bromelain, papain, and ficin either 0.005 M L-cysteine or 0.0167 M KCN, neutralized before use, was employed as the activator. Assays were always made in duplicate or triplicate, and the results were expressed in terms of per cent activity, taking the appropriate control run as reference.

DFP.[4] Three different preparations, A, B, and C, of DFP were used for studying their effect on casein hydrolysis by various proteinases. Preparation A was a product of Mann Research Laboratories, purchased in 1961. No lot number was given on the vial, which said "100% pure DFP." Preparation B was a specimen synthesized by Dr. Elias Awad at the University of Washington, Seattle, Wash. This was from the same lot of preparation with the one used in an earlier study by Murachi and Neurath (1960). Preparation C was a product of unknown origin. It had been stored among stock reagents in this laboratory. In a later part of the present experiment, preparation D was also employed in addition to the three preparations mentioned above. Preparation D was the product of Sumitomo Chemical Industries, Co., Ltd., Osaka, Japan,[5] and was found to show no inhibitory effect on bromelain as preparation A (see below). Since preparation D was available in large quantities, it was mostly employed for preparing DIP enzymes. With all of the four different preparations, the concentration of DFP was calculated by assuming that 10 μl of each preparation corresponds to 55 μmoles of DFP, regardless of the purity of individual preparations. In studying the effect of DFP on proteinase activity DFP was first dissolved in 1-propanol to make a 0.1 M solution, and varying amounts of the freshly prepared solution were added to the enzyme solution. Control runs were made with 1-propanol in these cases. In preparing DIP enzymes the liquid DFP was directly pipetted into the enzyme solution; a rapid dissolution into aqueous medium was attained under vigorous stirring. The rate of hydrolysis of DFP at alkaline medium was measured by using a Radiometer Model SBR2/SBU1/TTT1 autotitrator with NaOH as titrant. A glass electrode, Type 202B, and a calomel electrode, Type K401, were used.

Gas Chromatography.[6] Analysis was carried out on a Yanagimoto Model GCG-500 high-sensitivity gas chromatograph furnished with a hydrogen-flame ioniza-

[2] We are indebted to Dr. Ralph M. Heinicke for generous supply of this material.

[3] We are indebted to Dr. Herman Cohen for supplying this preparation as a gift to us.

[4] We are indebted to Drs. Hans Neurath, Kunihiko Saito, and Makoto Yamasaki for their cooperation in making various DFP preparations available to us for the present experiment.

[5] We are indebted to Dr. Seizaburo Yamaoka for generous supply of this product.

[6] We are indebted to Dr. Tsugio Takeuchi for performing gas chromatographic analyses.

tion detector. Celite 545, used as the solid support, was coated with 5% polyethylene glycol succinate and packed in a copper column 3 m long and 4 mm id. Nitrogen was used as the carrier gas under 1.2 kg/cm² and at a flow rate of 15 cc/min. The flow rate of hydrogen gas was 42 cc/min. Samples for analyses were dissolved in ethyl ether and 10 μl each was applied. The column temperature was maintained at 110 ± 2°.

Determination of SH Content. The "free" SH group of enzyme proteins was determined by the spectrophotometric titration with *p*-chloromercuribenzoate according to the method of Boyer (1954). A 2-ml sample containing 1 to 2 μmoles of stem bromelain was applied to a 1.12 × 20 cm (20 ml) column with Sephadex G-25 which had been washed with 0.05 M sodium acetate buffer saturated with nitrogen. The column was then washed with the same buffer under nitrogen stream and 3 ml of the effluent that followed the initial 6 ml was collected. The protein solution thus obtained free from L-cysteine or cyanide and DFP was diluted with 50–60 ml of 0.5 M sodium acetate buffer at pH 4 previously saturated with nitrogen, and immediately subjected to titration with *p*-mercuribenzoate at the same pH. A Hitachi Model EPB-U spectrophotometer was used at a wavelength of 250 mμ.

Other Methods. The pH was measured with a Hitachi Model M-4 glass electrode pH meter equipped with a temperature compensator. For gel filtration was used Sephadex G-25, medium, obtained from Pharmacia, Uppsala, Sweden. Phosphorus was determined by a modified Fiske-Subarrow method (Bartlett, 1959).

Results

Effect of DFP on the Caseinolytic Activities of Stem Bromelain, Papain, and Ficin. The enzyme was preincubated with either L-cysteine or KCN at pH 7.2 for 10 min at 30°. An aliquot of DFP solution in 1-propanol was then introduced to make final concentrations of 0.001 M DFP and 0.03 M cysteine or 0.1 M cyanide in a total volume of 1.0 ml. The preincubation was continued for an additional 30 min. The hydrolysis of casein was started by adding 5 ml of casein solution to the preincubation mixture. The reaction was allowed to continue for 10 min at 30°. The effects of three different preparations of DFP were examined. The results obtained are summarized in Table I. Activities of the DFP-treated enzymes are expressed as per cent activities, taking the specific activity of each enzyme preincubated only with the respective activator as reference.

It is apparent from the table that all three DFP preparations tested have no inhibitory effect on the casein hydrolysis by stem bromelain in the presence of cysteine. In the presence of cyanide, however, preparation A had no inhibitory effect while preparation C showed a marked inhibition which was not reversed by a later addition of excess cysteine to the reaction mixture. Preparation B gave a slight inhibition only in the presence of cyanide. The pattern of inhibition observed with stem bromelain holds also in the cases of papain and ficin. All three DFP preparations used were found to give a complete inhibition of *B. subtilis* proteinase activity under comparable conditions.

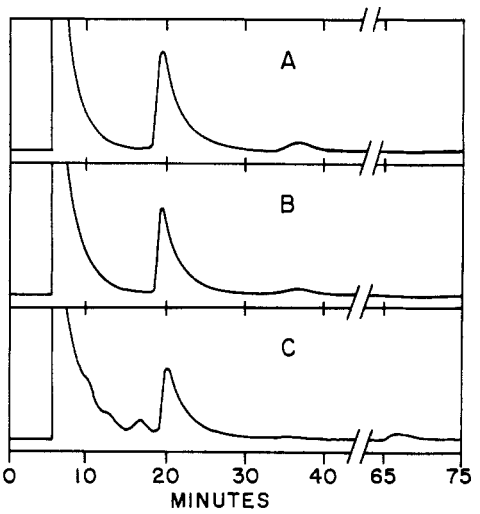

FIGURE 1: Gas chromatography of DFP preparations A, B, and C. Samples of DFP were dissolved in ethyl ether and 10 μl each of the solution was used for the analysis. The conditions for chromatographic runs are described in the text.

TABLE I: Effect of DFP on the Caseinolytic Activity of Stem Bromelain, Papain, and Ficin.[a]

Activator	DFP Prepn	Relative Activity[b] (%) Stem Bromelain	Papain	Ficin
0.03 M L-cysteine	A	102	108	100
	B	100	97	88
	C	100	106	107
0.1 M KCN	A	99	99	98
	B	79	68	74
	C	30	6	18
0.01 M KCN + 0.03 M L-cysteine[c]	B	82		
	C	36		

[a] Enzymes were preincubated with activator and 0.001 M DFP at pH 7.2 for 30 min at 30°. Casein hydrolysis was run at 30° and at pH 7.2. [b] The specific activity of each enzyme preincubated only with the respective activator is taken as 100%. [c] L-Cysteine (0.03 M) was added after stem bromelain had been preincubated with KCN and DFP for 30 min.

Gas Chromatography of DFP Preparations A, B, and C. Figure 1 shows tracings of the gas chromatograms obtained for three different DFP preparations. All of the three samples contained one major com-

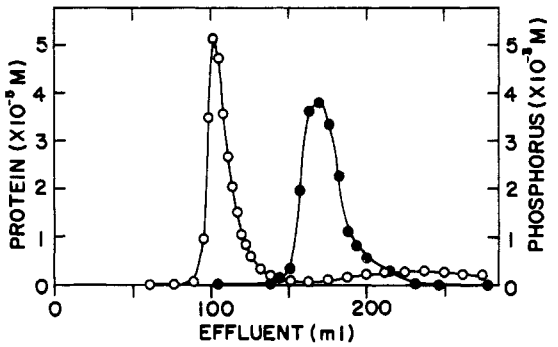

FIGURE 2: Gel filtration of the mixture after the reaction of DFP with stem bromelain. Stem bromelain (1 μmole) was incubated with 110 μmoles of DFP preparation D at 30° for 3 hr at pH 8.2. For gel filtration, a 250-ml column with Sephadex G-25 was used at pH 5.2; ○, protein concentration; ●, phosphorus concentration.

ponent which was supposed to be DFP. Preparations A and B contained small amounts of a component with longer retention times as compared to the major component. The chromatogram for preparation C indicated the presence of at least three components which appeared ahead of the peak for the major component. A prolonged run with preparation C revealed the presence of a small but significant amount of some component which appeared in a diffuse fashion with a retention time longer than 65 min.

Determination of SH Content after Treatment with DFP. To a solution of 2 μmoles of stem bromelain in 10 ml of 0.01 M phosphate buffer, pH 7.2, containing 0.03 M L-cysteine or 0.1 M KCN, was added 22 μmoles of DFP, and the mixture was incubated at 30° for 30 min. Solid ammonium sulfate was then added to 0.8 saturation at 0° and the precipitated protein was collected by centrifugation. The protein was dissolved in 2 ml of water and the SH content was determined according to the method described above. An aliquot of the protein solution was also subjected to the measurement of caseinolytic activity. Control runs were made by omitting DFP from the incubation mixture. The results obtained are shown in Table II, taking the specific activity of the cysteine-activated enzyme as 100%. In the absence of DFP, when cyanide was used as the activator, the specific activity toward casein was found to be significantly lower than that of the cysteine-activated enzyme, a finding in agreement with an earlier observation (Murachi and Neurath, 1960). This is explicable, as shown in Table II, in terms of a lower SH content of the cyanide-activated enzyme. Treatment of the enzyme with DFP preparation A in the presence of cysteine or cyanide caused neither inhibition nor decrease in SH content as compared to the enzyme preparation treated only with cysteine or cyanide, respectively. The enzyme treated with DFP preparation B or C showed a decreased activity toward casein, and the decrement can be accounted for by the parallel decrease in SH content of the enzyme preparation.

TABLE II: SH Content and Caseinolytic Activity of Stem Bromelain after Treatment with DFP.[a]

Activator	DFP	Mole of SH/Mole of Enzyme	Relative Activity[b] (%)
None	None	0.71	70
0.03 M L-cysteine	None	0.98	100
	A	0.98	103
0.1 M KCN	None	0.81	86
	A	0.83	83
	B	0.68	62
	C	0.20	25

[a] Stem bromelain was incubated with 0.0022 M DFP at pH 7.2 for 30 min at 30°. After precipitation of the enzyme protein with ammonium sulfate and gel filtration under an anaerobic condition, the SH content of the protein was determined spectrophotometrically. [b] The specific activity of the cysteine-activated enzyme is taken as 100%.

Preparation and Analysis of DIP Stem Bromelain. To a solution of 1 μmole of a stem bromelain in 3 ml of 0.25 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.2 were added 55 μmoles of DFP preparation D, and the mixture was incubated at 30°. The pH of the mixture was maintained by occasional addition of small amounts of 1 N NaOH. After an incubation for 1 hr, another 55 μmoles of the same DFP preparation was added and the incubation was continued for additional 2 hr. At the end of the incubation period, an aliquot was withdrawn for the assay of caseinolytic activity. The rest of the reaction mixture was applied to a 250-ml column with Sephadex G-25 which had been equilibrated with 0.05 M sodium acetate buffer at pH 5.2. The column was washed with the same buffer and the effluent was collected in 3-ml fractions. Each fraction was assayed both for the content of protein as determined by measuring the absorbancy at 280 mμ and for the content of phosphorus. A typical protocol is depicted in Figure 2. It is evident from the figure that the bed volume of the Sephadex column used was large enough to obtain a complete separation of protein fractions from DFP and its decomposition products which had been present in more than 100-fold molar excess in the reaction mixture.

The protein fractions were combined, and with small aliquots of the combined mixture analyses were carried out for protein concentration, phosphorus content, and caseinolytic activity. To the rest was added solid ammonium sulfate to saturation. The enzyme protein that precipitated was collected by centrifugation and dissolved in 3 ml of water. The solution was dialyzed *vs.* two changes of 2 l. of water for 30 hr at 4°. Aliquots were then withdrawn for assays from the dialyzed solu-

TABLE III: Phosphorylation of Stem Bromelain by DFP without Inhibition of Caseinolytic Activity.[a]

Enzyme and Treatment	Relative Activity[b] (%)	Moles of Phosphorus/ Mole of Protein
Stem bromelain before DFP treatment	100	0.00
After 3-hr incubation with DFP	96	..
After filtration through Sephadex G-25 gel	101	0.91
After 1st $(NH_4)_2SO_4$ precipitation and dialysis	96	0.91
After 2nd $(NH_4)_2SO_4$ precipitation and dialysis	93	0.94
Stem bromelain in 0.001 M $HgCl_2$ before DFP treatment	100 (2.5)	0.00
After 3-hr incubation with DFP	99 (2.1)	..
After filtration through Sephadex G-25 gel	102	1.10
After 1st $(NH_4)_2SO_4$ precipitation and dialysis	97	0.96
After 2nd $(NH_4)_2SO_4$ precipitation and dialysis	98 (8.9)	0.96
Trypsin before DFP treatment	100	0.00
After 3-hr incubation with DFP	0	..
After filtration through Sephadex G-25 gel	0	0.98
B. subtilis proteinase before DFP treatment	100	0.00
After 3-hr incubation with DFP	0	..
After filtration through Sephadex G-25 gel	0	1.02

[a] Incubation was carried out at 30° and at pH 8.2. Details of the procedure are described in text. Assays for stem bromelain were made in the presence of 0.005 M L-cysteine, except for those shown in parenthesis. Data in parenthesis were obtained without addition of cysteine. Cysteine was not used for trypsin and *B. subtilis* proteinase. [b] The specific activity of each enzyme before DFP treatment is taken as 100%.

tion and with the remainder the whole procedure of precipitation, redissolution, dialysis, and analysis was repeated. The results of these analyses are summarized in Table III.

In Table III are also presented data of an experiment in which the incubation of stem bromelain with DFP was carried out in the presence of 0.001 M $HgCl_2$. Stem bromelain is known to be strongly inhibited by mercuric ion and the inhibition is completely reversed by the addition of excess cysteine (Murachi and Neurath, 1960). The table also contains data of experiments with trypsin and *B. subtilis* proteinase.

As shown in Table III, stem bromelain reacts with DFP to lead the formation of a fully active, phosphorus-containing enzyme. Trypsin and *B. subtilis* proteinase were also phosphorylated with DFP but, as had been known (Hartley, 1960), the phosphorylated enzymes had no activity. The alkylphosphorylation of stem bromelain occurred as well even when the essential SH group of this enzyme had been blocked by mercuric ion. With stem bromelain and mercuri bromelain used as the starting materials, both phosphorus content and caseinolytic activity at each step of the procedures were found to remain practically constant, indicating that the phosphorus found in the protein fraction after gel filtration was firmly bound to the protein molecule.

Time Course of Phosphorylation of Stem Bromelain. Figure 3 shows the time course of phosphorus incorporation into the stem bromelain protein when the latter is incubated with DFP preparation D under the conditions similar to those employed for the experiments shown in Table III. To a solution of 7 μmoles of stem bromelain in 21 ml of 0.25 M Tris buffer at pH 8.2 were added 385 μmoles of DFP. After an incubation for 1 hr at 30° (arrow A in Figure 3), 275 μmoles of DFP was added to a 15-ml portion of the reaction

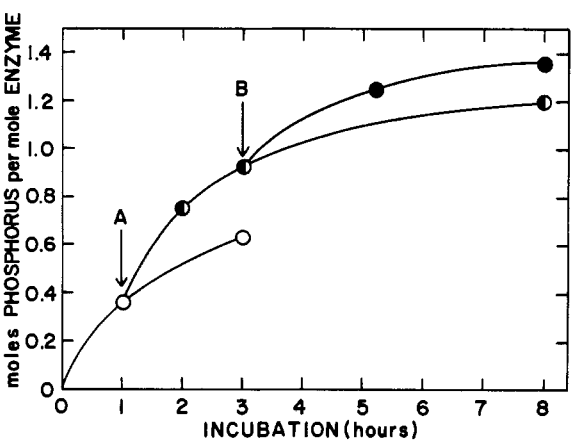

FIGURE 3: Time course of the phosphorylation of stem bromelain with DFP. DFP preparation D was used at 30° and at pH 8.2. Other experimental conditions are described in text; ○, with 55-fold molar excess of DFP added at time zero; ◐, with another 55-fold molar excess of DFP added at the first hour of incubation, arrow A; ●, with still another 55-fold molar excess of DFP added at the third hour of incubation, arrow B.

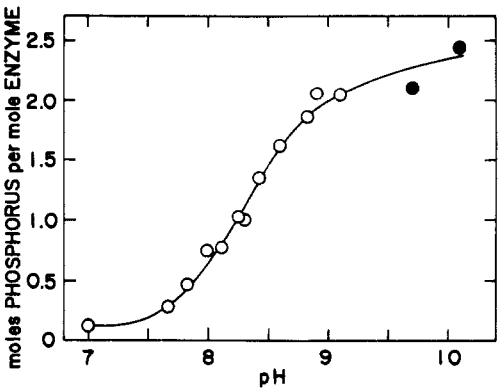

FIGURE 4: pH dependence of the phosphorylation of stem bromelain by DFP. Stem bromelain (1 μmole) was incubated with 110 μmoles of DFP preparation D at 30° for 3 hr at various pH values: ○, Tris buffer; ●, carbonate–bicarbonate buffer.

mixture, and the incubation was further continued. At the third hour from the initial addition of DFP (arrow B in Figure 3), 6 ml of the mixture was withdrawn and to this portion was added 110 μmoles of DFP. The reaction was continued until the eighth hour of incubation. The pH of the mixture was corrected after each addition of DFP. At given time intervals 2-ml aliquots were withdrawn from the incubation mixture and immediately applied to a 250-ml column with Sephadex G-25 gel in 0.05 M acetate buffer at pH 5.2. Gel filtration and analysis of phosphorus content of the protein were carried out as described above. As shown in the figure, the phosphorylation reaction proceeds so slowly that the reaction is not completed after a 3-hr incubation with 55-fold molar excess of DFP. Both the rate and the extent of phosphorylation were increased as more DFP was added to the reaction mixture.

pH Dependence of Phosphorylation of Stem Bromelain. The reaction mixture contained 1 μmole of stem bromelain and 55 μmoles of DFP preparation D in a total volume of 3 ml. Buffers used were 0.25 M Tris at pH 7.0–9.1 and 0.25 M sodium carbonate–bicarbonate at pH 9.7–10.1. After an incubation at 30° for 1 hr, 55 μmoles of DFP was added and the incubation was continued for additional 2 hr. The pH of the reaction mixture was maintained by occasional additions of 1 N NaOH. Gel filtration and analysis of phosphorus content were carried out as described above. The results obtained are shown in Figure 4. The phosphorylation shows a marked pH dependence, reaching a ratio of 2.4 moles of phosphorus/mole of enzyme protein at pH 10.1.

Hydrolysis of DFP in Alkaline Medium. The rates of hydrolysis of DFP preparation D at various alkaline pH values were determined by following the reactions in a pH-Stat assembly with NaOH as titrant. All the titration experiments were carried out in 0.1 M KCl and under nitrogen stream. The reactions satisfactorily obeyed first-order kinetics and the rate constant for each reaction was calculated, assuming that 2 equiv of H^+/mole of DFP was liberated by the hydrolysis. The calculated values are shown in Table IV. It is apparent from the table that the rate of hydrolysis is proportional to the concentration of OH^- in the medium. The data also indicate that only 3.3% DFP is decomposed after a 2-hr incubation at pH 9.0 and at 30°, while at pH 10.0 more than 31% disappears during the same period of time.

TABLE IV: Hydrolysis of DFP in Alkaline Medium.[a]

pH	First-Order Rate Constant (min^{-1}) At 25°[b]	At 30°[c]
9.0	...	2.9×10^{-4}
10.0	1.9×10^{-3}	3.1×10^{-3}
10.5	5.9×10^{-3}	...
11.0	1.9×10^{-2}	3.4×10^{-2}
11.2	3.3×10^{-2}	...
12.0	2.0×10^{-1}	...

[a] The rate of hydrolysis was measured in 0.1 M KCl and under nitrogen stream using a Radiometer pH-Stat assembly with NaOH as titrant. From the alkali consumption the first-order rate constant for each reaction was calculated, assuming that 2 equiv of H^+/mole of DFP was liberated by the hydrolysis. DFP preparation D was used. [b] The volume of the reaction mixture was 5.0 ml; the initial concentration of DFP was 5.5×10^{-2} M; 1 N NaOH was used as titrant. [c] The volume of the reaction mixture was 10.0 ml; the initial concentration of DFP was 2.75×10^{-3} M; 0.02 N NaOH was used as titrant for the experiment at pH 9.0 and 0.1 N NaOH for the experiments at other pH values.

Discussion

The present investigation has reconfirmed the results of our earlier experiment (Murachi and Neurath, 1960) that the bromelain-catalyzed hydrolysis of casein was not inhibited by DFP. Even in the absence of activator, DFP preparation D was found to give no inhibition (Table III). In addition, the present study has also reconfirmed the results of other investigators that some commercial preparations of DFP have inhibitory effects on plant proteinases when they are activated with cyanide (Masuda, 1959; Heinicke and Mori, 1959; Ota *et al.*, 1961; Ebata and Yasunobu, 1963). Preparation C used in the present study gave a marked inhibition of stem bromelain, papain, and ficin in the presence of cyanide. Once the inhibition had occurred, this was not reversed by the addition of excess cysteine to the medium. The titration of SH content of DFP-treated stem bromelain revealed a close parallelism between the proteinase activity and the SH content (Table II). This implies that the decrease in activity has resulted from the partial loss of the essential SH group of the en-

zyme. That such loss is not caused by DFP *per se* is evident because some DFP preparations have no inhibitory effect in the presence of cyanide as well as in the presence of cysteine. The most reasonable explanation of all the results reported in earlier and present papers may be that DFP *per se* has nothing to do with the essential SH group of stem bromelain, leaving the latter fully active, while some DFP preparations contain an impurity or impurities capable of attacking the essential SH group to form an irreversibly inhibited enzyme. The attack by the impurity must be of such a nature that it can be protected by excess cysteine but not by cyanide.

Using ficin, Gould *et al.* (1963) and Gould and Liener (1965) have found that there exists in some commercial preparations of DFP an impurity which reacts with SH groups. They fractionated a specimen of DFP from Aldrich Chemical Co., Milwaukee, Wis., by distillation under reduced pressure into four fractions. Fraction IV or the residue remaining in the distillation flask was found to represent the "impurity" of the original Aldrich DFP, showing a strong and irreversible inactivation of ficin with a parallel decrease in SH content of the enzyme protein. This fact is of great interest in view of the result of the gas chromatography in the present investigation. As shown in Figure 1, DFP preparation C has been found to contain impurities different from those found in preparations A and B. Preparation C is the one which showed a marked inhibition of stem bromelain in the presence of cyanide but not in the presence of cysteine; in this regard preparation C closely resembles Aldrich DFP used for the study of ficin (Gould and Liener, 1965). Preparative gas chromatographic separation of these impurities would have provided further information concerning the chemical nature of the inhibitor, but this was not attempted because of the limited amount of preparation C left in this laboratory.

The earlier report from this laboratory (Murachi, 1963) was the first demonstration of the occurrence of a reaction of DFP with enzyme protein which did not result in inactivation but did cause phosphorylation. The enzymes with which such reaction was shown to occur were stem bromelain, papain, and Taka-amylase A. We have further found that egg white lysozyme can also be phosphorylated with a great excess of DFP at an alkaline pH above 9 and the phosphorylated lysozyme shows no decrease in activity toward glycol chitin used as the substrate (Murachi and Inagami, 1963). Subsequently Gould *et al.* (1963) confirmed the occurrence of such reaction with ficin and papain. The experiments with stem bromelain are described in detail in the present paper. A careful study was made to rule out a possibility that the enzyme protein merely adsorbed DFP or its derivative onto its surface. All the possible means for separation were employed which were thought to be useful in making a protein free from low molecular weight contaminants without denaturing effect. Thus, gel filtration, ammonium sulfate precipitation, and dialysis were used in combination and repeatedly. As a result of such experiment (Table III), it is concluded that a true alkylphosphorylation had occurred with no inhibition. A further support of this conclusion has been provided by isolating and characterizing *O*-DIP-L-tyrosine from the enzymatic hydrolysate of the phosphorylated stem bromelain (Murachi and Inagami, 1963). The details of the latter experiment will be described in a subsequent communication (Murachi *et al.*, 1965).

It has been known that DFP phosphorylates chymotrypsin and other proteinases and esterases of animal origin (see, for example, the review by Hartley, 1960). The present paper describes that DFP also phosphorylates stem bromelain. The phosphorylation of stem bromelain, however, differs from that of chymotrypsin in the following four points. (1) The phosphorylation of stem bromelain does not accompany inactivation of the enzyme. (2) Incorporation of phosphorus into stem bromelain may exceed 1 mole of phosphorus/mole of enzyme protein, while in the case of chymotrypsin a ratio of 1:1 has been reported. (3) The phosphorylation of stem bromelain readily occurs only at an alkaline pH higher than 8. (4) A much higher molar ratio of DFP to enzyme protein is required to obtain an appreciable phosphorylation of stem bromelain. Chymotrypsin can be readily phosphorylated at neutral pH with a slight molar excess of DFP. These differences must reflect the differences in the mechanism and in the site of phosphorylation of both enzymes. In contrast to the fact that chymotrypsin is phosphorylated at the specific serine hydroxyl group which is supposed to be in the active site, the phosphorylation of stem bromelain occurs somewhere other than the cysteine SH group which is essential for activity. The phosphorylation of stem bromelain was found to occur as well even when the essential SH group had been blocked by mercuric ion (Table III). Further investigations in this laboratory (Murachi and Inagami, 1963; Murachi *et al.*, 1965) have revealed that the site of phosphorylation in stem bromelain molecule is tyrosine residue or residues. Gould and Liener (1965) described a phosphorylation of ficin with DFP without inhibition, and they obtained indirect evidence to indicate that the serine (and/or threonine) residues are the most likely sites of phosphorylation.

The pH dependence of phosphorylation of stem bromelain may provide some information concerning the amino acid residues that are involved in the reaction. As shown in Figure 4, the plot for the pH dependence seems to conform to a sigmoid curve, and the pattern is not incompatible with a postulation that ionized tyrosine hydroxyl groups are the reactants. However, one may interpret the pattern of the plot only in a qualitative way: care must be taken in any quantitative interpretation because of the following two reasons.

(1) Since the rate of hydrolysis of DFP in alkaline medium has been known to increase logarithmically with pH (Table IV), the concentration of DFP in the reaction mixture with stem bromelain is subject to a rapid decrease during incubation at a pH as high as 10. It has also been demonstrated by the present experiment (Figure 3) that the phosphorylation depends on the concentration of DFP. These facts suggest that

the value in Figure 4 for the phosphorus incorporation at a pH beyond 9 must be lower than the value which one could have obtained if the concentration of DFP were kept constant during incubation at that pH.

(2) Even at a pH below 9 where the alkali hydrolysis of DFP during incubation is only limited so that one may assume a constant concentration of DFP (Table IV), the value in Figure 4 for the phosphorus incorporation does not represent the maximal extent of the reaction attainable at that pH. This is because the phosphorylation reaction was not rapid enough to reach its completion within 3 hr at 30° (Figure 3).

The true pH dependence would be found by studying the initial rate of phosphorylation as a function of pH by maintaining the DFP concentration constant throughout the incubation period or, alternatively, by carrying out the experiment at a given initial concentration of DFP and correcting the rate constant for the decrement of DFP concentration due to alkali hydrolysis that occurs concurrently. Such experiments were actually conducted in studying the alkylphosphorylation by DFP of *N*-acetyl-L-tyrosine amide and egg white lysozyme (Murachi and Inagami, 1963).

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