INACTIVATION OF MELON PROTEINASE BY DIISOPROPYL FLUOROPHOSPHATE

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INACTIVATION OF MELON PROTEINASE BY DIISOPROPYL FLUOROPHOSPHATE

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Abstract

Melon proteinase is inhibited strongly by diisopropyl fluorophosphate and considerably by phenylmethanesulfonyl fluoride, specific inhibitors of serine enzymes.

The reaction of the enzyme with diisopropyl fluorophosphate at pH 7.1 resulted in the incorporation of 0.82 atom of phosphorous into the enzyme molecule.

Introduction

Melon proteinase is a proteolytic enzyme isolated from the sarcocarp of melon (Cucumis Melo L. var. Prince). The enzyme is strongly inhibited by diisopropyl fluorophosphate, but not by inhibitors of cysteine enzymes or metal enzymes (*1*). Therefore, the enzyme seems to be a serine-type proteinase.

In structural studies of active site of plant proteinases, in contrast to the extensively studied cysteine proteinases, relatively little is known about the serine proteinases. In the first case, Shaw and Wells (*2*) have reported that the sequence immediately around the reactive serine of a carboxypeptidase from bean leaves differs from that in trypsin and chymotrypsin or subtilisin.

The present study describes the inhibition of melon proteinase by inhibitors of serine enzymes and the incorporation of phosphorous into the enzyme molecule.

Materials and Methods

Melon proteinase was purified as described previously (*1*). The enzyme fractions from the gel-filtration were collected, and dialyzed exhaustively against distilled water, and lyophilized.

Diisopropyl fluorophosphate (DFP) was a product of BDH Chemicals, Ltd., England. Amidol (2,4-diaminophenol hydrochloride) was purchased from Nakarai Chemicals, Ltd., Kyoto. Diisopropyl fluorophosphate treated trypsin was a product of Worthington Biochemical Corp., U.S.A. Phenylmethanesulfonyl fluoride (PMSF) was a product of Sigma Chemical Co., U.S.A.

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Preparation of diisopropyl phosphoryl melon proteinase — Melon proteinase, 150 mg (3 μmole), was reacted with DFP (30 μmole) in 5 ml of 0.05 M Tris-HCl buffer, pH 7.6, for 5 hours at 30°. In order to remove the excess reagent, the mixture was passed through a column (1.5×100 cm) of Sephadex G-75 equilibrated with 0.1 M acetic acid. The protein was monitored by measuring the absorbance at 280 nm. The fractions containing the protein were collected and lyophilized after the eluate pooled was diluted with water several times.

Estimation of phosphorous — Diisopropyl phosphoryl melon proteinase, 30 mg (0.6 μmole), was placed in a Pyrex test tube (1.8×18 cm), and 1.0 ml of 60% perchloric acid was added. The mixture was heated on a sand bath cautiously. The temperature was raised slowly until white fumes were evolved, when the evolution of fumes ceased the temperature was raised to about 230° and held constant for one hour. After cooling, 1.0 ml of the reducing reagent containing 0.4 g of Amidol and 8 g of sodium sulfite in 100 ml of water was added slowly. Then 1.0 ml freshly prepared solution of 3.3% ammonium molybdate in water was added. After thorough mixing, the test tube was heated in a boiling water bath for 10 min. After cooling, the solution was made up to a 10 ml with water and the absorbance at 720 nm was measured against blank solution containing all reagents. A calibration curve was constructed for quantities of monopotassium phosphate.

Assay of proteinase — Proteolytic activity was measured by the method of Kunitz (*3*), with casein as a substrate.

Results and Discussion

Inhibition by DFP and PMSF — The enzyme was completely inactivated by incubation with 0.5 mM DFP for 20 min and considerably inhibited with 0.5 mM PMSF, as shown in Table 1. It was observed that the inactivation of melon proteinase by DFP occurred more rapidly than did that by PMSF.

Hayashi et al. (*4*) have already shown that DFP reacts stoichiometrically with the hydroxyl group of a serine residue of carboxypeptidase Y, producing an inactivated enzyme which contains one diisopropyl phosphoryl group per enzyme molecule.

Table 1. Inhibition of melon proteinase by DFP and PMSF

Addition	Concentration[a] (mM)	Relative activity[b] (%)
DFP	0.5	0
PMSF	0.5	55

a Concentrations are those in the preincubation mixture.
b Activity of a control with no addition was taken as 100%.
The enzyme (12 μg) was preincubated in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.1, containing inhibitor for 20 min at 37°. After preincubation, 0.5 ml of 2% casein in the same buffer was added to the mixture and the activity was assayed by the Kunitz method.

It is known that sulfonyl halides, such as PMSF, react with many of the serine enzyme, such as chymotrypsin, trypsin, and acetylcholinesterase, to sulfonylate the hydroxyl group of a serine residure in the active site and thereby inhibit enzymic activity. In this respect they are analogous to the organophosphates, such as DFP.

Reaction of DFP — The DFP-treated proteinase was sufficiently separated from excess DFP by gel-filtration on Sephadex G–75. The determination of DFP eluted from a column was done by the measurement of the inhibitory action on the caseinolysis using trypsin as a proteinase. The protein fraction pooled had not proteolytic activity against casein, therefore melon proteinase was completely inactivated by DFP. The mixture of the diisopropyl phosphoryl proteinase and perchloric acid showed a brown color in the beginning of heating, finally its color disappeared. The amount of the incorporation of phosphorous in the preparation was 0.82 atom per molecule of molecular weight 50,000. This value was corrected for a 20% loss, based upon the loss from authentic diisopropyl phosphoryl trypsin in a parallel experiment.

In many successful studies where the organophosphorus compounds have been used as specific modification agents, the organophosphorus compounds react stoichiometrically with a hydroxyl group of a particularly reactive serine in the native susceptible enzyme to yield a product which consist of the enzyme and the substituent moiety in a 1:1 ratio (*5*), however, the analytical results in this preparation were about 20% lower than theoretical value. As the reactivity of DFP is lost after denaturation of protein, it seems that the enzyme used here contained a certain amount of denaturated protein. It has been reported that the melon proteinase is labile for lyophilization, which caused loss of more than 10% of the proteolytic activity (*1*).

References

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