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# SPECIFICITY OF THE SERINE PROTEASE INHIBITOR, PHENYLMETHYLSULFONYL FLUORIDE

V. Sekar and James H. Hageman

Department of Chemistry
New Mexico State University
Las Cruces, New Mexico 88003

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## Summary

Clarified cell-free extracts were prepared from rapidly dividing Bacillus subtilis cells and from rabbit liver cells. These extracts were treated with [$^3$H]-phenylmethylsulfonyl fluoride (PMSF) and analyzed by electrophoresis in isoelectric focusing polyacrylamide gels or detergent gels. Not less than 14 proteins in the B. subtilis extracts and not less than 15 proteins in rabbit liver extracts reacted covalently with PMSF. These results suggest that PMSF is not as specific for serine proteases as sometimes supposed, and its effects in physiological experiments should be interpreted with caution.

## INTRODUCTION

Phenylmethylsulfonyl fluoride has become a very widely used covalent serine protease inhibitor since its introduction by Fahrney and Gold in 1963 (1). Although it is often considered to be highly specific for serine proteases, PMSF is known to inhibit other classes of enzymes; some instances of this are cited by James in a recent study on the stability of PMSF (2). In addition, papain (3), malonyltransacylase (4) and γ-glutamyl transpeptidase (5) are reported to be inhibited by PMSF.

The extracellular bacillopeptidase F produced by B. subtilis 168 was found to undergo autolysis. Many of the autolytic fragments were found to react with [$^3$H]-PMSF (Carolyn A. Roitsch, Ph.D. dissertation, New Mexico State University). In the course of trying to purify the single, PMSF-sensitive protease present in crude, cell-free extracts of Bacillus subtilis (6), we attempted to use [$^3$H]-PMSF to label and stabilize the enzyme during purification. The large number of protein species which became labeled made this procedure impractical and is the subject of this report.

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## MATERIALS AND METHODS

Electrophoresis and isoelectric focusing supplies were purchased from the following chemical companies: acrylamide and bis-acrylamide, electrophoresis grade, from Aldrich; ammonium persulfate from Baker; N,N,N',N'-tetramethylethylenediamine (TEMED) from Sigma; sodium dodecylsulfate (SDS) and NonidetP-40 (NP-40) from BDH (purchased through Gallard-Schlesinger); Ampholines, pH ranges 5-7, 4-6 and 3.5-10, from LKB and urea, ultra pure, from Schwarz-Mann.

Trizma base, glycine, β-mercaptoethanol, L-tryptophan and D-glucose were all obtained from Sigma. Complete counting cocktail (3a70B) and 5-ml low-potassium mini glass vials were purchased from Research Products International Corp. [$^3$H]-phenylmethylsulfonyl fluoride (PMSF), sp. act: 3.91 mCi/ml (0.162 mCi/mg), was prepared by New England Nuclear. $H_2O_2$ (30%) was a product from Fisher.

B. subtilis 168, a tryptophan auxotroph first described by Spizizen (7), was cultured as previously described (6). Liver tissue was obtained from a cervically dislocated New Zealand white male rabbit which had been killed one hour prior to excision. The liver tissue was suspended in sonication buffer (8), pH 7.4, and chilled immediately in an ice bath. Following homogenization in a Sorvall Omni-mixer at 4°C, a cell-free extract was prepared by centrifugation at 18.000 x g for 1 hr.

Two 50-ml cultures of B. subtilis 168 were grown to 120 Klett units, and the cells obtained from each culture were suspended in an equal volume of sonication buffer and were broken in a 5-ml French Pressure cell (Aminco) at 16,000 psi. From the pooled lysates clear soluble extract was prepared according to the method of O'Farrell (8).

To 1 ml of the B. subtilis 168 extract were added 10 µl of [$^3$H]-PMSF and to another were added the same amount of [$^3$H]-PMSF (but in 20 µl) which had been hydrolyzed by treatment with 0.1 M NaOH and reneutralized to pH 7 with HCl. The latter sample served as a control to detect any possible non-covalent associations. Both samples were incubated at 37°C for 1 hr. with shaking and dialyzed for 12 hr. against 0.05 M $NaHCO_3$, pH 10, with 5 changes of buffer.

Isoelectric focusing and SDS-polyacrylamide gels were made 11 cm long and electrophoreses were done as described in reference (8). Following electrophoresis, gels were sliced into 2-mm slices in a Gilford gel slicer. Individual slices were placed in 5-ml mini glass vials and were digested with 0.1 ml (each) of 30% $H_2O_2$ for 4 hrs. at 77°C. After cooling, they were mixed with 3 ml (each) of 3a70B complete counting cocktail and were counted in a Beckman LS-100C liquid scintillation counter.

Rabbit liver extracts were treated with [$^3$H]-PMSF and analyzed in exactly the same manner as the B. subtilis extracts.

## RESULTS AND DISCUSSION

When crude cell-free extracts prepared from logarithmically growing B. subtilis were exposed to [$^3$H]-PMSF, numerous proteins became covalently modified by this reagent. Figure 1 shows that upon separation by isoelectric focusing, not less than 14 proteins contained significant amounts of label. The proteins in the control samples treated with base-inactivated [$^3$H]-PMSF were labeled to a much lower extent which suggests the labeling is not due to non-covalent association.

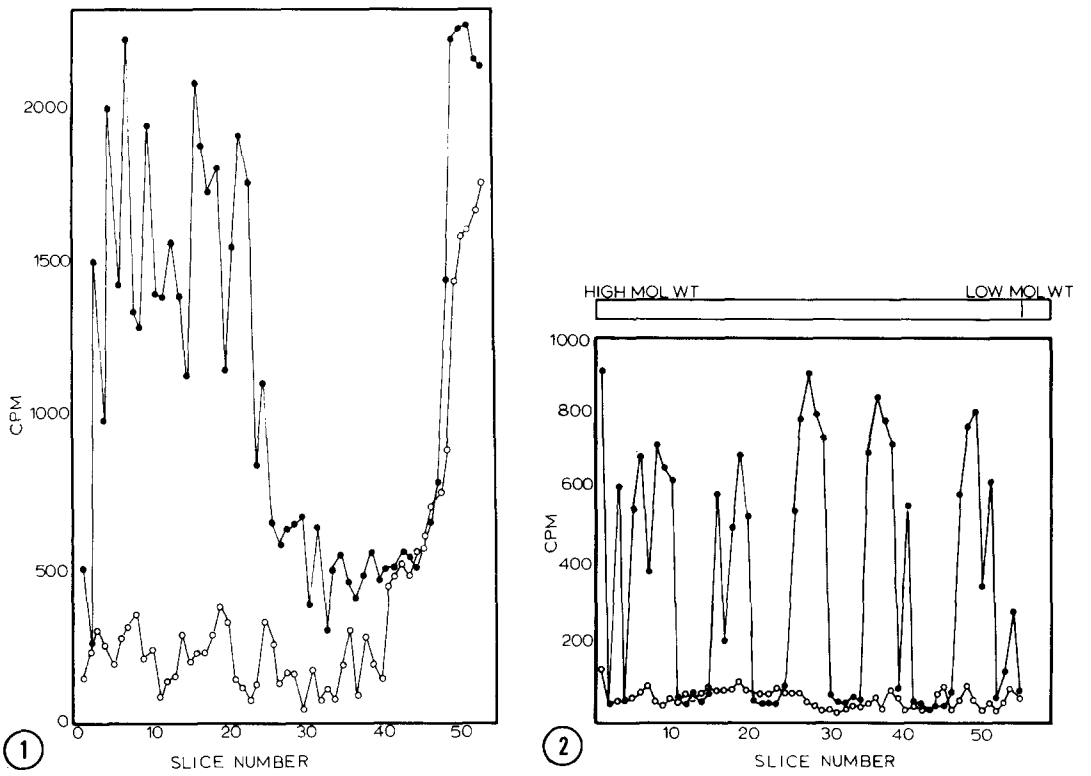
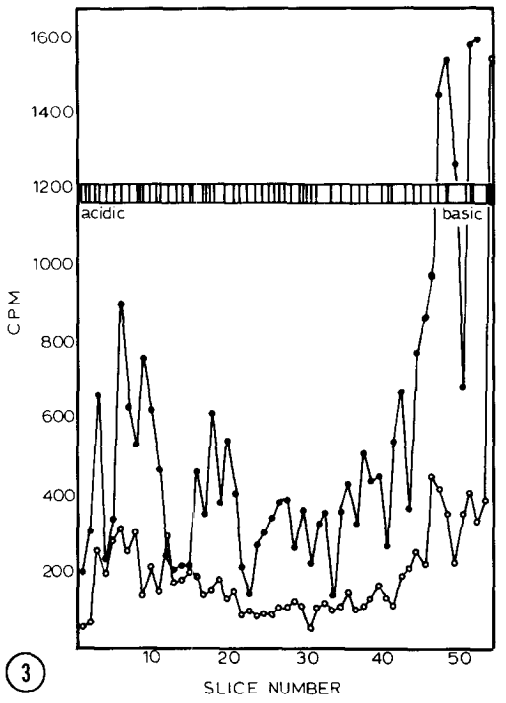



Figure 1. Separation of B. subtilis Proteins Exposed to [$^3$H]-PMSF on Isoelectric Focusing Polyacrylamide Gels. Solid circles, extract treated with [$^3$H]-PMSF and dialyzed before electrophoresis; open circles, extract treated with base-hydrolyzed [$^3$H]-PMSF and dialyzed before electrophoresis. The pH of the left-hand end of the gel is 4.25 and increases to 8.3 at the right-hand end.

Figure 2. Separation of B. subtilis Proteins Exposed to [$^3$H]-PMSF on SDS-10% Polyacrylamide Gels. Solid circles, extract treated with [$^3$H]-PMSF and dialyzed before electrophoresis; open circles, extract treated with base-hydrolyzed [$^3$H]-PMSF and dialyzed before electrophoresis. The top of the gel is on the left. The position of the dye front at the end of electrophoresis is shown in the bar at the top of the figure.

To be certain that the label was indeed covalently attached, the B. subtilis extracts were also subjected to electrophoresis on polyacrylamide gels in the presence of the detergent, sodium dodecyl sulfate. As Figure 2 shows, not less than 11 polypeptides became labeled.

To determine whether these labeling patterns were peculiar to B. subtilis, a crude cell-free extract was prepared from rabbit liver, and the number of



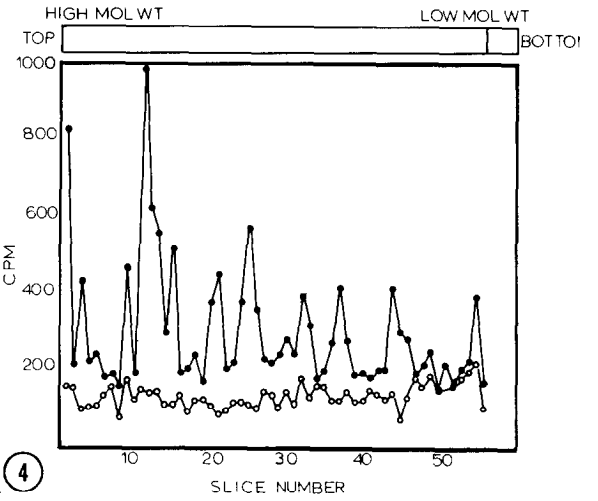




Figure 3. Separation of Rabbit Liver Proteins Exposed to [$^3$H]-PMSF on Isoelectric Focusing Gels. Symbols and conditions are the same as for Figure 1. The bar near the top of the figure shows the appearance of the gel after staining for proteins with Coomassie Blue R-250 dye.

Figure 4. Separation of Rabbit Liver Proteins Exposed to [$^3$H]-PMSF on SDS-10% Polyacrylamide Gels. Symbols and conditions are the same as for Figure 2. The top of the gel is on the left. The position of the dye front at the end of electrophoresis is shown in the bar at the top of the figure.

covalently labeled proteins was determined by electrophoresis on isoelectric focusing gels (Figure 3) and on detergent gels (Figure 4). Both analyses revealed that 15-16 proteins became labeled with tritium. It should be stressed that in all cases the number of bands seen must be a minimal estimate in that the 2-mm slices often contained many more than one protein as can be seen from the protein staining pattern of the gel shown in the upper part of Figure 3.

In view of the reactive nature of PMSF (2), these observations are perhaps not unexpected. It seems unlikely to us, in view of the literature cited, that all of the labeled proteins observed here are serine proteases or esterases. Indeed, in Escherichia coli it has been reported that PMSF induced a dramatic decline in ATP pools when the medium became depleted of glucose (9).

In recent years PMSF has been used not only in studies of purified serine proteases but also in complex or whole-cell studies (10-14). In the latter cases the aims were to determine or to block the action of serine protease(s) in various physiological processes. In light of the observations reported here, we believe inferences regarding protease function based on treatment of such systems with PMSF should be made with caution.

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