BBA 65923

# STUDIES ON *STREPTOMYCES GRISEUS* PROTEASE

## III. PURIFICATION OF TWO DFP-REACTING ENZYMES

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(Received February 24th, 1969)

SUMMARY

Two DFP-sensitive enzyme components, with esterase activity against *p*-nitrophenyl acetate, have been prepared from a commercial preparation of *Streptomyces griseus* protease (Pronase). After initial separation on CM-cellulose, one enzyme was purified by chromatography on phosphate cellulose, on TEAE-cellulose and on Sephadex G-50 and the other by chromatography on CM-cellulose and on Sephadex G-50. A high degree of purity was demonstrated by polyacrylamide-gel electrophoresis and by an equimolar incorporation of phosphorus from DFP into the proteins. The molecular weights of the enzymes were determined to be 16 000 and 18 000, respectively.

## INTRODUCTION

In a recent paper[1] from this laboratory, the separation of three DFP-inactivable enzymes from a commercial preparation of *Streptomyces griseus* protease (Pronase, Calbiochem) was described. One enzyme with esterase activity against α-*N*-benzoylarginine ethyl ester (BAEE) was extensively purified, and the tripeptide sequence (Asp–Ser-*P*–Gly) at its DFP-reacting site was determined[2]. The other enzymes with esterase activity against *p*-nitrophenyl acetate (PNPA) were shown by a radiochemical method to give phosphopeptide patterns identical with that of the BAEE hydrolase after inactivation with [$^{32}$P]DFP and partial acid hydrolysis. This result indicated that all three enzymes have the same amino acid sequence at the active site.

An account is given in the present paper of the further purification of the two enzymes hydrolyzing PNPA. The enzymes are denoted as PNPA hydrolase I and II from their order of appearance on CM-cellulose chromatography of Pronase[1].

Abbreviations: BAEE, α-*N*-benzoylarginine ethyl ester; PNPA, *p*-nitrophenyl acetate; GPNA, *N*-glutaryl-L-phenylalanine *p*-nitroanilide; Ser-*P*, *O*-phosphorylserine.

## EXPERIMENTAL

### *Protein and phosphorus determinations*

The protein of the chromatographic fractions was estimated by absorbance measurement at 280 nm in a Beckman DU spectrophotometer. A protein solution with an absorbance of 1.0 (1-cm light path) contains by definition one $A_{280\ nm}$ unit of protein per ml.

The absorbance at 280 nm of 1% solutions containing the purified enzymes was estimated after dissolving a weighed amount of salt-free, lyophilized enzyme which was 9 for PNPA hydrolase I and 13 for PNPA hydrolase II. These values were used when the molar incorporation of phosphorus from DFP into the enzymes was determined.

The phosphorus content of the DFP-inactivated enzyme was measured as orthophosphate by the method of MARTIN AND DOTY[3] after digestion of the protein by the method of GRUNBAUM *et al.*[4].

### *Enzyme determination*

The activity against PNPA and BAEE (both substrates purchased from Sigma Chemical Co., St. Louis, Mo.) of the chromatographic fractions was measured as previously described[1]. One $A_{400\ nm}$ unit of PNPA hydrolase activity is defined as the amount of enzyme causing the hydrolysis of 1 $\mu$mole of PNPA per min at 25° (pH 7.0). One $A_{250\ nm}$ unit of BAEE hydrolase activity is defined as the amount of enzyme hydrolyzing 1 $\mu$mole of BAEE per min at 25° (pH 7.5) (*cf.* ref. 1).

To study the enzyme activity remaining after incubation with DFP for different times, the activity assay method had to be modified, since the 30-min assay time originally used was too long. PNPA hydrolase I activity was estimated with *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) as substrate[5], and PNPA hydrolase II activity was measured with PNPA as follows.

The GPNA substrate solution was prepared by dissolving 20 mg of the substance (purchased from Mann Research Laboratories) in 2 ml of methanol. The solution was made up to 100 ml with 0.05 M Tris–HCl (pH 7.5), making the substrate solution 0.5 mM. For each assay, 2.5 ml of substrate solution at 25° and 0.5 ml of enzyme solution were mixed. After 5 min, the reaction was terminated by the addition of 0.5 ml of 30% (v/v) acetic acid, and the amount of *p*-nitrophenol liberated was estimated at 410 nm in a Beckman B spectrophotometer.

PNPA hydrolase II was assayed at 25° after diluting an appropriate amount of enzyme solution to 3.1 ml with 0.05 M Tris–HCl (pH 7.5). The reaction was started by the addition of 25 $\mu$l of 35.4 mM PNPA in acetonitrile, and the color development was followed at 400 nm in a Beckman B spectrophotometer for 3 min. A control for correction of the spontaneous hydrolysis of PNPA was run in parallel.

### *Chromatographic procedures for the purification of PNPA-hydrolases I and II*

Ten g of Pronase (Calbiochem, Los Angeles, Calif., U.S.A., lot no. 45550) was chromatographed on CM-cellulose at pH 5.0, as described previously[1]. Two main protein peaks appeared with the gradient, the first containing PNPA-hydrolase activity (PNPA-hydrolase I) as well as BAEE-hydrolase activity, and the second peak containing PNPA-hydrolase activity (PNPA-hydrolase II).

The two activities of the first peak were separated by chromatography on phosphate cellulose at pH 4.2 (ref. 1). The fractions containing PNPA-hydrolase activity were pooled and concentrated by ultrafiltration according to EVERALL AND WRIGHT[6] with dialysis tubing (Union Carbide, Chicago, Ill., U.S.A.) of 23/32 inch inflated diameter as filtering membrane. The concentrated enzyme solution was chromatographed on a Sephadex G-50 column in equilibrium with 0.01 M Tris-HCl buffer (pH 8.9) also containing 0.01 M NaCl. The fractions containing PNPA-hydrolase activity were pooled, and applied to a TEAE-cellulose column equilibrated and eluted with the same buffer. Finally, PNPA-hydrolase I was chromatographed on Sephadex G-50 in equilibrium with 0.01 M calcium acetate-acetic acid buffer (pH 5.0).

For purification of PNPA-hydrolase II, the fractions of the second peak of the CM-cellulose chromatography of Pronase were pooled, and dialyzed against 0.04 M calcium acetate-acetic buffer (pH 6.0). After dialysis for 24 h at 4°, the enzyme solution was chromatographed on a CM-cellulose column in equilibrium with the same buffer. Elution was performed with a linear gradient from 0.04 M to 0.15 M calcium ion concentration in the aforementioned buffer. The fractions with PNPA-hydrolase activity were pooled, and concentrated by ultrafiltration as described above, and finally chromatographed on a Sephadex G-50 column equilibrated with 0.01 M calcium acetate-acetic acid buffer (pH 5.0). In order to remove traces of BAEE-hydrolase activity, the last step was repeated.

*Polyacrylamide-gel electrophoresis*

The purity of the two PNPA hydrolases was investigated by polyacrylamide-gel electrophoresis (performed by Dr. Bo Löfqvist, Institute of Biochemistry, Lund, Sweden). The gel (6% with 10% cross-linking) was prepared in 0.38 M borate buffer (pH 7.0) containing 0.01 M $Ca^{2+}$. Electrophoresis was performed in the same buffer for 1.5 h (cathodic migration) at 32 V/cm and about 19 mA. The current was allowed to

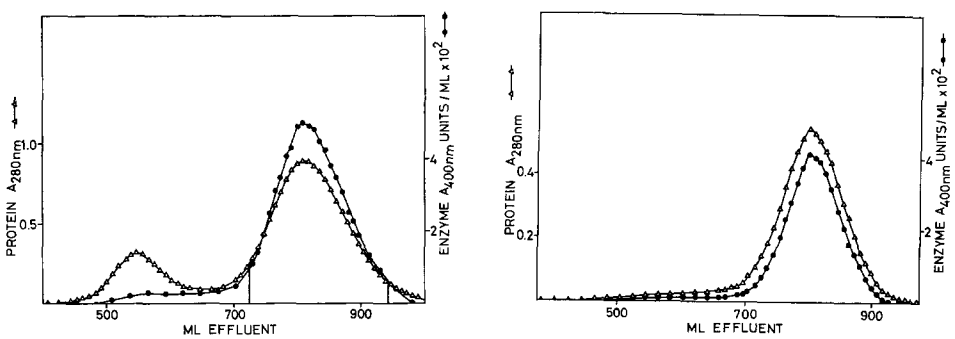


Fig. 1. Chromatography of pooled and concentrated PNPA hydrolase I fractions from phosphate cellulose (Fig. 2, ref. 1) on Sephadex G-50. Column dimensions: 6.6 cm × 34.4 cm. Elution performed with 0.01 M Tris–HCl (pH 8.9) containing 0.01 M NaCl. 10-ml fractions collected every 10 min. Material pooled as indicated by vertical lines in the figure and chromatographed on TEAE-cellulose. △—△, protein, absorbance at 280 nm; ●—●, PNPA hydrolase activity, $A_{400\ nm}$ units/ml.

Fig. 2. Chromatography of pooled and concentrated fractions from TEAE-cellulose chromatography on Sephadex G-50. Column dimensions: 6.6 cm × 34.4 cm. Elution performed with 0.01 M calcium acetate–acetic acid buffer (pH 5.0). 10-ml fractions collected every 5 min. All symbols are the same as those in Fig. 1.

flow through the gels for 30 min before the sample was applied. The protein was stained in Coomassie blue after fixation in 12.5% trichloroacetic acid[7].

*Time-course of inactivation with DFP*

The purified PNPA hydrolases were titrated with 1 M Tris to pH 7.5, were diluted with 0.05 M Tris–HCl (pH 7.5) to a protein concentration of approx. 11 $\mu$M for PNPA hydrolase I and 15 $\mu$M for PNPA hydrolase II, and were incubated with [$^{32}$P]DFP at a final concentration of 30 $\mu$M and 50 $\mu$M, respectively. At different time intervals, enzyme activity was measured as described above, and amounts of [$^{32}$P]Ser-*P* were determined as reported in the previous paper[1].

*Molecular weight determination*

Sedimentation analyses of the purified enzyme components were performed at 20° in a Beckman Model E analytical ultracentrifuge at 52 640 rev./min. The molecular weights were determined by the approach-to-equilibrium method according to EHRENBERG[8]. A partial specific volume of 0.71 for PNPA hydrolase I and 0.72 for PNPA hydrolase II, based on preliminary amino acid analyses, were used in the calculations.

## RESULTS AND DISCUSSION

*Purification of PNPA hydrolase I*

Fig. 1 shows Sephadex G-50 chromatography of PNPA hydrolase I (pH 8.9). The esterase activity was eluted after about 800 ml with the main protein peak. A small protein peak containing about 20% of the total protein content, but without any appreciable amount of PNPA hydrolase activity, was eluted in front of the main peak. Since the ratio of activity to protein was not constant over the PNPA hydrolase peak, it had to contain other protein constituents and was applied to a TEAE-cellulose

TABLE I

RECOVERY OF PROTEIN AND PNPA HYDROLASE ACTIVITY DURING PURIFICATION OF PNPA HYDROLASE I FROM *S. griseus* PROTEASE

The specific activities are given as ratios of PNPA hydrolase activity to amount of protein, expressed as $A_{400\ nm}$ units and $A_{280\ nm}$ units, respectively.

| *Preparation* | *Total protein content ($A_{280\ nm}$ units)* | *PNPA hydrolase activity (total $A_{400\ nm}$ units)* | *Specific activity ($\times 10^2$)* |
|---|---|---|---|
| Dialyzed starting material | 7550 | 62* | — |
| Pooled material from CM-cellulose | 1560 | 26 | 1.7 |
| Pooled material from phosphate cellulose | 270 | 13 | 4.8 |
| Pooled material from G-50 (pH 8.9) | 116 | 6.6 | 5.7 |
| Pooled material from TEAE-cellulose | 83 | 5.0 | 6.0 |
| Recovered material from G-50 (pH 5.0) | 58 | 4.6 | 7.9 |

* The total activity of the starting material includes PNPA hydrolases I and II as well as activity of material appearing with the front of the effluent of the chromatography on CM-cellulose.

column in equilibrium with the same buffer as in the G-50 column. The PNPA hydrolase appeared with the front of the effluent, while about 20% of the total protein was retained on the column. The protein retained on the column could be eluted by increased NaCl concentration and was found to be completely inactive with respect to PNPA hydrolase activity.

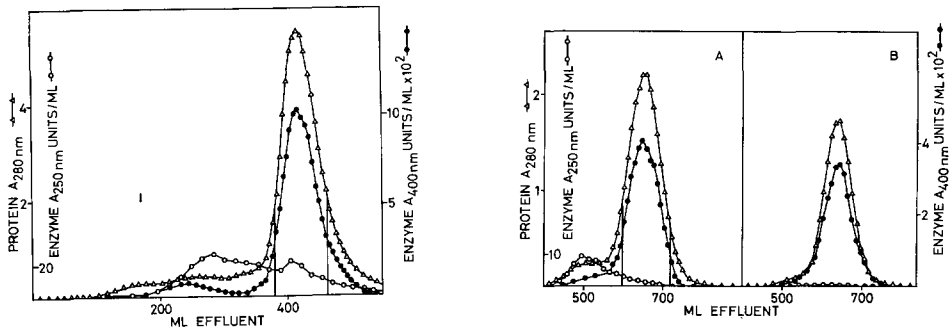


Fig. 3. Chromatography of pooled PNPA hydrolase II fractions from CM-cellulose (Fig. 1, ref. 1) on CM-cellulose. Column dimensions: 3.8 cm × 13.2 cm. Elution performed with a linear gradient from 0.04 M to 0.15 M calcium acetate–acetic acid buffer (pH 6.0). Elution volume: 400 ml collected in 5–6-ml fractions every 4 min. Gradient start at the top of the column is indicated in the figure by an arrow. Material pooled as indicated by vertical lines and chromatographed on Sephadex G-50. △—△, protein, absorbance at 280 nm; ●—●, PNPA hydrolase activity, $A_{400\ nm}$ units/ml; ○—○, BAEE hydrolase activity, $A_{250\ nm}$ units/ml.

Fig. 4. (A) Chromatography of pooled and concentrated PNPA hydrolase II from the chromatogram in Fig. 3 on Sephadex G-50. (B) Rechromatography of pooled and concentrated fractions from Fig. 4A on the same column. Column dimensions: 6.6 cm × 34.4 cm. Elution performed with 0.01 M calcium acetate–acetic acid buffer (pH 5.0). 10-ml fractions collected every 5 min. All symbols are identical with those in Fig. 3.

Fig. 2 shows PNPA hydrolase I fractions from the TEAE-cellulose chromatographed on Sephadex G-50. Protein and enzyme activity were eluted together as one symmetrical peak.

Table I shows the recoveries of protein and enzyme activity at different stages of the purification of PNPA hydrolase I.

*Purification of PNPA hydrolase II*

The last protein peak from the CM-cellulose chromatography (pH 5.0) of the Pronase[1] was first chromatographed on CM-cellulose (pH 6.0) as shown in Fig. 3. A small amount of protein containing BAEE hydrolase activity was eluted before te main component, which appeared at a buffer concentration of approx. 0.07 M.

The fractions of the main protein peak were pooled as indicated, were concentrated to about 25 ml and were applied to a Sephadex G-50 column at pH 5.0. As seen in Fig. 4A, a small amount of BAEE hydrolase was separated from the PNPA hydrolase, indicating a difference between the enzymes with respect to size or shape. After rechromatography in the same system, shown in Fig. 4B, an apparently homogeneous preparation of PNPA hydrolase II was obtained.

Table II shows the recoveries of protein and enzyme activity at different stages of the purification of PNPA hydrolase II.

TABLE II

RECOVERY OF PROTEIN AND PNPA-HYDROLASE ACTIVITY DURING PURIFICATION OF PNPA HYDROLASE II FROM *S. griseus* PROTEASE

The specific activities are given as ratios of PNPA-hydrolase activity to amount of protein, expressed as $A_{400\ nm}$ units and $A_{280\ nm}$ units, respectively.

| *Preparation* | *Total protein content ($A_{280\ nm}$ units)* | *PNPA-hydrolase activity (total $A_{400\ nm}$ units)* | *Specific activity ($\times 10^2$)* |
|---|---|---|---|
| Dialyzed starting material | 6900 | 50* | |
| Pooled material from CM-cellulose (pH 5.0) | 607 | 10 | 1.6 |
| Pooled material from CM-cellulose (pH 6.0) | 306 | 6.0 | 2.0 |
| Pooled material from first Sephadex G-50 | 195 | 3.8 | 1.9 |
| Recovered material from second Sephadex G-50 | 130 | 2.7 | 2.1 |

* See footnote of Table I.

*Purity of the PNPA hydrolases*

A test of purity was performed by electrophoresis on polyacrylamide gel, shown in Fig. 5. After staining, PNPA hydrolase I showed only one narrow band. PNPA hydrolase II showed, however, two very weak bands in addition to the main component. The purity of PNPA hydrolase II was estimated to be more than 95%. As a reference, crude Pronase from the same batch was run.

The ultracentrifuge experiments showed only one symmetrical boundary. The

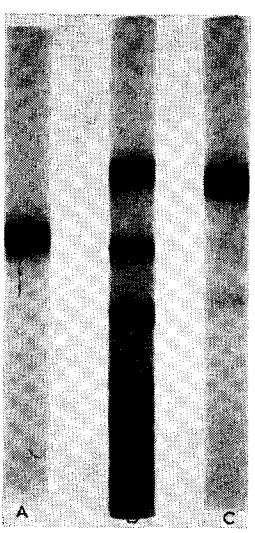


Fig. 5. Polyacrylamide-gel electrophoresis of PNPA hydrolase I (A), commercial Pronase (B) and PNPA hydrolase II (C). Amounts of protein: 8 μg of PNPA hydrolase I, 114 μg of Pronase and 17 μg of PNPA hydrolase II. Electrophoresis was performed in 0.38 M borate buffer (pH 7.0), containing 0.01 M $Ca^{2+}$, at 32 V/cm and about 19 mA for 1.5 h (cathodic migration; upwards in the figure). The protein was stained in Coomassie blue after fixation in 12.5% trichloroacetic acid.

TABLE III

TIME COURSE OF ACTIVITY DECREASE AND PHOSPHORUS INCORPORATION ON INCUBATION OF A PNPA HYDROLASE I PREPARATION WITH [$^{32}$P]DFP

The purified enzyme from Fig. 2 incubated for different times with 30 $\mu$M [$^{32}$P]DFP at pH 7.5 and 25°.

| *Incubation time (min)* | *Enzyme activity (% of initial activity)* | *[$^{32}$P]Ser-P (% of final amount)* |
|---|---|---|
| 0 | 100 | 0 |
| 5 | 60 | 41 |
| 11 | 34 | 68 |
| 20 | 15 | 85 |
| 40 | 2 | 99 |
| 80 | 0 | 100 |

molecular weights were determined to be approx. 16 000 and 18 000 for PNPA hydrolase I and II, respectively.

In order to further study the purity of the enzymes, the molar incorporation of phosphorus from DFP into the purified enzymes was determined. A molar incorporation of 1.0 could be calculated for both PNPA hydrolases.

The correlation between incorporation of $^{32}$P from [$^{32}$P]DFP, determined as [$^{32}$P]Ser-*P*, and the loss of enzyme activity after incubation for different times was also studied. The results are given in Tables III and IV.

TABLE IV

TIME COURSE OF ACTIVITY DECREASE AND PHOSPHORUS INCORPORATION ON INCUBATION OF A PNPA HYDROLASE II PREPARATION WITH [$^{32}$P]DFP

The purified enzyme from Fig. 4B incubated for different times with 50 $\mu$M [$^{32}$P]DFP at pH 7.5 and 25°.

| *Incubation time (min)* | *Enzyme activity (% of initial activity)* | *[$^{32}$P]Ser-P (% of final amount)* |
|---|---|---|
| 0 | 100 | 0 |
| 5 | 56 | 42 |
| 10 | 33 | 63 |
| 20 | 15 | 82 |
| 40 | 7 | 92 |
| 80 | 3 | 99 |
| 180 | 0 | 100 |

The enzymes were inactivated in parallel to the binding of phosphorus. It can be concluded from the incorporation studies that each enzyme has one active site which is modified during the reaction with DFP.

The data obtained on the purity of the two enzymes show that both enzymes

are sufficiently purified to permit further studies of structure as well as of the mechanism of action.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. L. Engström and Dr. Ö Zetterqvist for invaluable discussions throughout this work. I am also indebted to Mrs. M. Säfström and Miss A. Holst for excellent technical assistance and to Mr. H. Pertoft for performing the ultracentrifuge experiments. This investigation was supported by grants from the Swedish Medical Research Council (Project No. 40X-665), the Delegation for Applied Medical Defence Research (Projects No. 89/66 and 27/67), the Medical Faculty of the University of Uppsala, and from Svenska Sällskapet för Medicinsk Forskning.

REFERENCES

1 S. WÄHLBY, *Biochim. Biophys. Acta*, 151 (1968) 394.
2 S. WÄHLBY AND L. ENGSTRÖM, *Biochim. Biophys. Acta*, 151 (1968) 402.
3 J. B. MARTIN AND D. M. DOTY, *Anal. Chem.*, 21 (1949) 965.
4 B. W. GRUNBAUM, F. L. SCHAFFER AND P. L. KIRK, *Anal. Chem.*, 24 (1952) 1487.
5 B. F. ERLANGER, F. EDEL AND A. COOPER, *Arch. Biochem. Biophys.*, 115 (1966) 206.
6 P. H. EVERALL AND G. H. WRIGHT, *J. Med. Lab. Technol.*, 15 (1958) 209.
7 A. CHRAMBACH, R. A. REISFELD, M. WYCKOFF AND J. ZACCARI, *Anal. Biochem.*, 20 (1967) 150.
8 A. EHRENBERG, *Acta Chem. Scand.*, 11 (1957) 1257.