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STUDIES ON *STREPTOMYCES GRISEUS* PROTEASE

I. SEPARATION OF DFP-REACTING ENZYMES AND PURIFICATION OF ONE OF THE ENZYMES

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SUMMARY

1. By chromatography on CM-cellulose, a commercial preparation of *Streptomyces griseus* protease (Pronase, Calbiochem) has been shown to contain at least three DFP-reacting components. One of the components has esterase activity against benzoylarginine ethyl ester, and the others against *p*-nitrophenyl acetate.

2. The enzyme with esterase activity against benzoylarginine ethyl ester has been purified by consecutive chromatography on CM-cellulose and phosphate cellulose. The purity of the enzyme has been studied by sedimentation analysis, which showed only one moving boundary. A high degree of purity was also indicated by an equimolar incorporation of phosphorus from DFP into the enzyme.

INTRODUCTION

Pronase, a commercial preparation of *Streptomyces griseus* protease, was shown by starch zone electrophoresis to contain at least four different fractions with proteolytic activity. One of these fractions is inactivated by DFP (ref. 1).

It was preliminarily reported from this laboratory[2] that an enzyme fraction with esterase activity against *N*-benzoyl-L-arginine ethyl ester (BAEE) could be obtained from Pronase by chromatography on CM-cellulose. The esterase activity was abolished by DFP, and the fact that [^{32}P]phosphorylserine ([^{32}P]SerP) could be isolated from an acid hydrolysate of the [^{32}P]DFP-inactivated preparation showed that the enzyme contained reactive serine residues. However, the [^{32}P]DFP-treated protein could be resolved into two ^{32}P-labelled fractions by chromatography on TEAE-cellulose, suggesting the presence of another DFP-reacting component in addition to the BAEE-hydrolase[2]. The main object of the present investigation was to separate the DFP-reacting enzymes of Pronase and, if possible, to identify them,

Abbreviations: BAEE, α-*N*-benzoyl-L-arginine ethyl ester; PNPA, *p*-nitrophenyl acetate; SerP, phosphorylserine.

as well as to purify at least one enzyme component, to establish the structure of its active site.

By consecutive chromatography on CM-cellulose and phosphate cellulose, three DFP-reacting enzyme fractions were separated. Two of the fractions were shown to have *p*-nitrophenyl acetate (PNPA)-hydrolase activity, and the third had BAEE-hydrolase activity. Indications were obtained of a fairly high purity of the BAEE-hydrolase. This component was used for the work described in the following paper[3].

EXPERIMENTAL

Enzyme activity assays

Esterase activity was measured against BAEE and PNPA. Both substrates were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and were used without further purification. The activity against BAEE (0.25 mM in 0.05 M Tris–HCl buffer (pH 7.5)) was determined according to SCHWERT AND TAKENAKA[4]. The measurements were made at 25° in a Beckman spectrophotometer, Model DU, at 250 mμ. 3.0 ml of substrate solution and 0.1 ml of enzyme solution in appropriate dilution were used for each assay. One $A_{250m\mu}$ unit (Figs. 1, 2 and 3) is defined as the amount of enzyme hydrolysing 1 μmole of BAEE per min under the conditions used.

The PNPA-hydrolase activity was determined according to the method of HUGGINS AND LAPIDES[5] with some modifications. The substrate solution was prepared by diluting 1 ml of a stock solution of PNPA in abs. methanol (35.4 mM) with distilled water to a final vol. of 100 ml. 3.9 ml of 0.1 M Tris–HCl buffer (pH 7.0) and 0.1 ml of enzyme solution in appropriate dilution were mixed and kept at 25°. The enzyme reaction was then started by the addition of 1 ml of substrate solution. The liberation of *p*-nitrophenol was measured at 400 mμ after 30 min at 25°. One $A_{400m\mu}$ unit (Figs. 1, 2 and 3) is defined as the amount of enzyme causing the hydrolysis of 1 μmole of PNPA per min.

Proteolytic activity was determined at pH 7.5 by the method of ANSON[6], with the modification that digestion was performed at 25° with 0.5 ml of haemoglobin substrate and 0.1 ml of enzyme solution. The reaction was terminated after 10 min with 1 ml of 5% trichloroacetic acid (w/v). The precipitated protein was removed by centrifugation. 1 ml of the supernatant was made alkaline by the addition of 2 ml of 0.5 M NaOH. The colour developed after the addition of the Folin–Ciocalteu reagent to the alkaline supernatant was measured in a Beckman B spectrophotometer at 660 mμ. One $A_{660m\mu}$ unit (Figs. 1, 2 and 3) is defined as the amount of enzyme giving a colour development per min of digestion equivalent to the colour obtained from 1 μmole of tyrosine.

Protein and phosphorus determinations

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

The phosphorus content of the DFP-inactivated enzyme protein was measured as orthophosphate by the method of MARTIN AND DOTY[7], after digestion of the protein by the method of GRUNBAUM, SCHAFFER AND KIRK[8].

Molecular weight determination

Sedimentation analysis of the purified enzyme component was performed at 20° in a Beckman Model E Analytical Ultracentrifuge at 52 640 rev./min. The molecular weight was determined by the Archibald method, as modified by EHRENBERG[9]. A partial specific volume of 0.72, based on a preliminary amino acid analysis, was used in the calculations.

Chromotography of Pronase

Ten g of Pronase (Calbiochem, Los Angeles, Calif., U.S.A., lot no. 45 550) was dissolved in 100 ml of 0.01 M calcium acetate–acetic acid buffer* (pH 5.0). A small amount of insoluble material was removed by centrifugation at 9000 × *g* for 20 min at 0°. The supernatant was dialysed against the same buffer overnight at 4°.

The dialysed Pronase was chromatographed at 4° on a CM-cellulose column (Serva Entwicklungslabor, Heidelberg, Germany, lot. no. 12 065), equilibrated with 0.01 M calcium acetate–acetic acid buffer (pH 5.0). Elution was performed with a linear gradient of 0.01 to 0.20 M calcium acetate–acetic acid buffer (pH 5.0).

The BAEE-hydrolase fractions were pooled and dialysed against 0.005 M calcium acetate–acetic acid buffer (pH 4.2). The pooled fraction was then chromatographed at 4° on a phosphate cellulose column (Whatman, P 70 powder, W. and R. Balston, England, lot. no. 171) in equilibrium with the buffer. The enzyme was eluted with a linear gradient of 0.005 to 0.10 M buffer concentration.

Incubation of Pronase fractions with [^{32}P]DFP

[^{32}P]DFP was obtained from the Radiochemical Centre, Amersham, England, and unlabelled DFP from Sigma Chemical Co., St. Louis, Mo., U.S.A. Before use, the labelled DFP was diluted with propane-1,2-diol to a concentration of 0.01 M.

1 ml of each fraction tested in the chromatograms was titrated with 1 M Tris to pH approx. 7.5 (indicator paper) and incubated overnight at room temperature with 5 mM [^{32}P]DFP (about 10^6 counts/min per μmole). The inactivated enzyme was precipitated with 1/4 vol. of 25% trichloroacetic acid–0.01 M silicotungstic acid. In order to achieve as complete a precipitation as possible, carrier bovine serum albumin (Sigma) was added to a final protein concentration of approx. 2% immediately prior to precipitation. The precipitate was washed 3 times with HCl–acetone (1 ml of conc. HCl in 250 ml of acetone) and dried *in vacuo*. After hydrolysis with 2 M HCl for 20 h at 100°, [^{32}P]SerP was isolated by chromatography on Dowex 50W-X8 columns eluted with 0.01 M HCl, as previously described[2]. The amounts of [^{32}P]SerP isolated were determined and used as a measure for DFP-reacting material of the fractions.

Time course of inactivation with DFP

The purest fraction with BAEE-hydrolase activity from the second phosphate cellulose chromatography (Fig. 3) was titrated with 1 M Tris to pH 7.5, diluted to a protein concentration of approx. 15 μM and incubated with [^{32}P]DFP at a final concentration of 50 μM. At different time intervals, BAEE-hydrolase activity and amounts of [^{32}P]SerP were determined as described above.

* The molarity of the calcium acetate–acetic acid buffers is given with respect to the concentration of calcium acetate.

RESULTS

Chromatography of 10 g of Pronase on CM-cellulose is shown in Fig. 1.

A large protein peak with no BAEE-hydrolase activity but with a small amount of PNPA-hydrolase activity appeared at the elution front. The second major protein peak, which contained all the BAEE-hydrolase and a large amount of PNPA-hydrolase, was eluted at a buffer concentration of approx. 0.05 M. Judging from the lack of

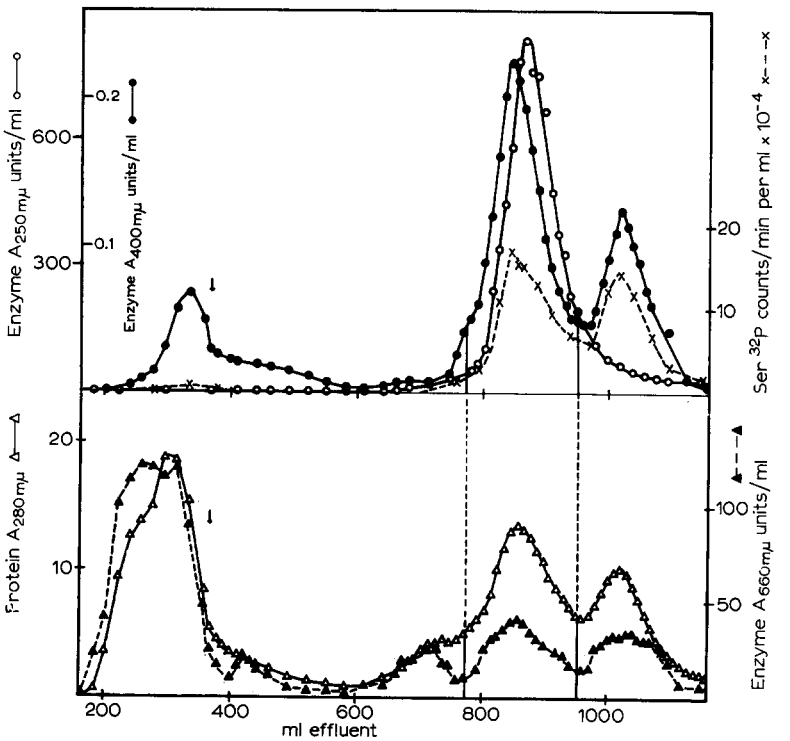
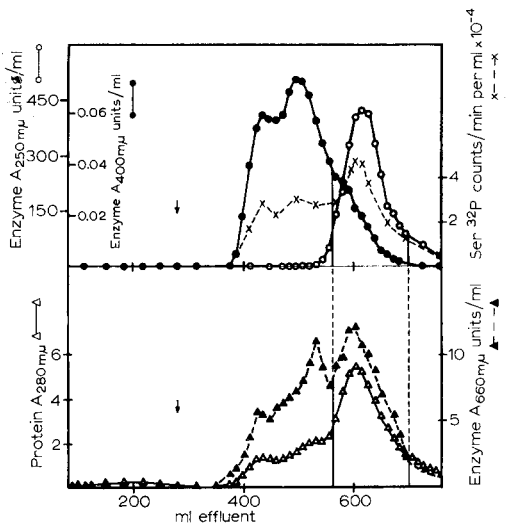


Fig. 1. Chromatography of 10 g of Pronase on CM-cellulose. Column dimensions: 3.8 cm × 20.3 cm. Elution with a linear gradient from 0.01 to 0.20 M calcium acetate–acetic acid buffer (pH 5.0). Elution volume: 1 l collected in 10-ml fractions every 6 min. Gradient started at the arrow. Material pooled as indicated by vertical lines in the figure and chromatographed on phosphate cellulose. △—△, protein, absorbance at 280 mμ; ▲—▲, proteolytic activity, $A_{660\ m\mu}$ units per ml; ○—○, BAEE-hydrolase activity, $A_{250\ m\mu}$ units per ml; ●—●, PNPA-hydrolase activity, $A_{100\ m\mu}$ units per ml; ×—×, [^{32}P]SerP, counts/min per ml × 10^{-4}.

parallelism between the two esterase peaks, they are apparently associated with different proteins. The third protein peak, eluted at a buffer concentration of approx. 0.08 M, contained PNPA-hydrolase activity. All the fractions had proteolytic activity.

The amounts of [^{32}P]SerP isolated from the different fractions show that DFP-reacting proteins were present only in the two most retarded protein peaks, and seemed to be associated with the esterases. However, the first protein peak also had some PNPA-hydrolase activity, but yielded a very small amount of [^{32}P]SerP after incubation with [^{32}P]DFP.



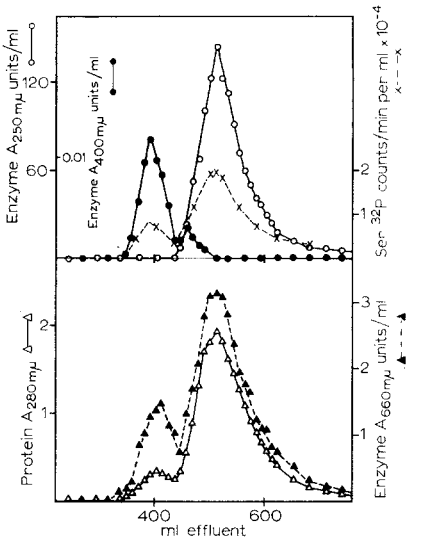


Fig. 2. Chromatography of pooled fractions from Fig. 1 on phosphate cellulose. Column dimensions: 3.0 cm × 15.0 cm. Elution with a linear gradient from 0.005 to 0.10 M calcium acetate–acetic acid buffer (pH 4.2). Elution volume: 600 ml collected in 10-ml fractions every 6 min. Gradient started at the arrow. Material pooled as indicated by vertical lines in the figure. All symbols are the same as in Fig. 1.

Fig. 3. Rechromatography of pooled fractions from Fig. 2 on phosphate cellulose under identical conditions.

The material with the BAEE-hydrolase was pooled as indicated in Fig. 1, and was further chromatographed on a phosphate cellulose column (Fig. 2).

Most of the PNPA-hydrolase activity was eluted together with some protein shortly after starting the gradient, at a buffer concentration of approx. 0.04 M. It was not completely separated from the BAEE-hydrolase. The latter component was eluted at a buffer concentration of approx. 0.07 M. The DFP-incorporating ability was approximately parallel to the esterase activities, as seen from the [^{32}P]SerP curve.

The fractions containing the BAEE-hydrolase were pooled, as indicated in Fig. 2,

TABLE I

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

Fraction	*Total protein content* ($A_{280\ m\mu}$ *units*)	*BAEE-hydrolase activity*	
		Total $A_{250\ m\mu}$ *units*	*Ratio* $A_{250\ m\mu}$ *units to* $A_{280\ m\mu}$ *units*
Dialysed starting material	6900	110 600	16.0
Pooled material from CM-cellulose	1700	75 300	44.3
Pooled material from first phosphate cellulose column	500	34 700	69.4
Recovered material from second phosphate cellulose column	200	13 600	68.0

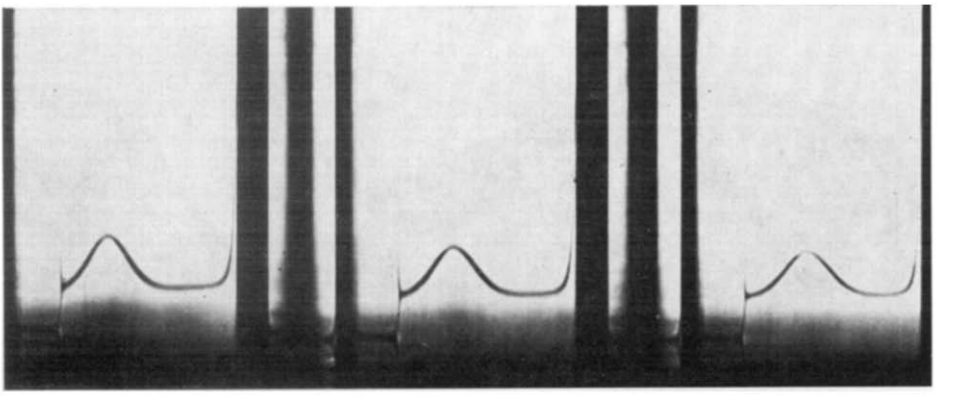


Fig. 4. Sedimentation analysis of the purified BAEE-hydrolase. Protein concentration: 10 mg/ml. Bar angle: 60°. Sedimentation from left to right. Exposures taken 107, 123 and 139 min after reaching full speed (52 640 rev./min).

and rechromatographed in the same system (Fig. 3). Almost complete separation of the two esterases was achieved by this procedure.

The small amount of overlapping PNPA-hydrolase was mostly eluted before the main component. The fractions of the BAEE-hydrolase peak showed approximately constant ratios of esterase activity and amount of SerP to protein content.

Table I summarizes the yields of a BAEE-hydrolase purification. Approx. 12% of the BAEE-hydrolase of the starting material was recovered in this particular preparation; however, yields of up to 35% of the theoretical were obtained on other occasions.

Fig. 4 shows the result of a sedimentation analysis of the purified BAEE-hydrolase component. Only one symmetric boundary, with a sedimentation constant of 2.3 S, was obtained, indicating a homogeneous protein. The molecular weight determination gave a value of approx. 19 000.

In order to further study the purity of the enzyme, the molar incorporation of phosphorus from DFP into the purified enzyme was determined from nitrogen and

TABLE II

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

The purest enzyme fraction from Fig. 3. incubated for different times with 50 μM [^{32}P]DFP at pH 7.5 and 25°.

Incubation time (min)	*BAEE-hydrolase (% of initial activity)*	*[^{32}P]SerP (% of final amount)*
0	100	0
10	66	36
20	52	55
40	26	68
80	9	87
180	0	100

phosphorus analyses, following inactivation with DFP. This preparation gave a molar ratio phosphorus to protein of 0.90, using a molecular weight of 19 000 and a nitrogen content of 16%. Other preparations gave values ranging from 0.90 to 1.00.

Table II shows the correlation between incorporation of ^{32}P, determined as [^{32}P]SerP, and the loss of esterase activity after incubation for different times with [^{32}P]DFP. It can be seen that the enzyme was inactivated parallel to the binding of phosphorus.

DISCUSSION

By chromatography on cellulose ion exchangers, a commercial preparation of Pronase has been demonstrated to contain at least three enzyme components reacting with DFP. All three components seem to be esterases, one of which is active against BAEE and the other two against PNPA. They all yield SerP on acid hydrolysis after DFP inactivation.

NARAHASHI AND YANAGITA[10] studied proteases and peptidases of Pronase by chromatography on CM-cellulose in a way similar to the first chromatographic step used in the present investigation. They reported two enzyme fractions which were inactivated by DFP.

A rough calculation of the total amount of protein-bound phosphorus, isolated as [^{32}P]SerP, from the chromatographic results presented in Fig. 1—under the assumption that the mean molecular weight of the crude preparation of Pronase is 20 000 and that 25% of the protein-bound phosphorus is obtained as [^{32}P SerP (ref. 11)—indicates that no less than approx. 40% of the protein reacts with DFP. Calculations from the data in Table I show that about 2/3 of this protein is the BAEE-hydrolase and the rest seems to consist of the PNPA-hydrolases.

The fact that the esterase activity of the purified BAEE-hydrolase disappears at the same rate as phosphorus from DFP is introduced into the enzyme, shows that the active site is modified during the reaction. Further, the incorporation of one mole of phosphorus per mole of enzyme is consistent with the presence of one active site per molecule.

These results, as well as the sedimentation analysis, indicate that the BAEE-hydrolase component is sufficiently purified to permit a study of the amino acid sequence around the reactive serine residue of the enzyme.

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