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STUDIES ON *STREPTOMYCES GRISEUS* PROTEASE

II. THE AMINO ACID SEQUENCE AROUND THE REACTIVE SERINE RESIDUE OF DFP-SENSITIVE COMPONENTS WITH ESTERASE ACTIVITY

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SUMMARY

1. A highly purified esterase preparation from *Streptomyces griseus* protease (Pronase), active against benzoylarginine ethyl ester, was inactivated with [^{32}P]DFP and hydrolysed with HCl. From the acid hydrolysate, the dipeptides Asp–[^{32}P]SerP and [^{32}P]SerP–Gly, as well as the tripeptide Asp–([^{32}P]SerP, Gly), were isolated. This shows that the amino acid sequence around the reactive serine residue of the enzyme is Asp–Ser–Gly.

2. Two other esterase components from Pronase, active against *p*-nitrophenyl acetate, were inactivated with [^{32}P]DFP and hydrolysed in the same manner. On paper electrophoresis at pH 3.5, identical patterns of ^{32}P-labelled peptides were obtained from all three enzyme components. This indicates that the three esterases have the same amino acid sequence at the DFP-reacting site.

INTRODUCTION

Many hydrolytic enzymes are inactivated by DFP (ref. 1). In all these enzymes, the inactivation is believed to be caused by phosphorylation of a particular serine residue at the active site. Studies of the amino acid sequence around the reactive serine have revealed different types of sequence. In all animal enzymes investigated so far, the sequence Asp(or Glu)–Ser–Gly(or Ala) has been demonstrated. On the other hand, this sequence has not been found in bacterial and mould proteases[1,2], with one recent exception[3].

In the preceding paper[4], a commercial preparation of *Streptomyces griseus* protease (Pronase) was shown to contain at least three DFP-sensitive enzyme components with esterase activity which, on inactivation with [^{32}P]DFP and acid hydrolysis, yielded [^{32}P]SerP. The purification of one of the components, a α-N-benzoyl-L-arginine ethylester (BAEE) hydrolase, is described. The other two DFP-sensitive

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

enzymes are *p*-nitrophenyl acetate (PNPA) hydrolases, which can be separated from each other as well as from the BAEE-hydrolase by chromatography.

The BAEE-hydrolase preparation obtained was considered to be sufficiently purified for a study of the amino acid sequence around the reactive serine residue. This enzyme was therefore inactivated by [^{32}P]DFP and partially hydrolysed with acid. ^{32}P-labelled phosphopeptides were isolated from the hydrolysate and identified. It could be concluded from the results that the amino acid sequence around the reactive serine residue in the BAEE-hydrolase is Asp–Ser–Gly. The two PNPA-hydrolases seem to have the same sequence.

In a preliminary paper[5] from this laboratory, indications of a similarity between the active sites of chymotrypsin and a BAEE-hydrolase preparation from Pronase were reported. However, the BAEE-hydrolase used in that work had only been chromatographed on CM-cellulose, and could be expected to contain one of the PNPA-hydrolases detected later[4]. This may explain why two labelled enzyme fractions were obtained on TEAE-cellulose chromatography of [^{32}P]DFP-inactivated enzyme[5].

EXPERIMENTAL

Enzyme purification

BAEE-hydrolase was purified from Pronase (*S. griseus* protease) as described in the preceding paper[4].

Analytical methods

The ninhydrin reaction was performed according to the method of MOORE AND STEIN[6], using a final vol. of 1.2 ml (0.2 ml of sample, 0.2 ml of ninhydrin reagent and 0.8 ml of 50% ethanol). The ninhydrin reagent of LEWIS[7] was used to spray the dried papers from the electrophoretic and chromatographic experiments.

Total phosphorus was determined as orthophosphate by the method of MARTIN AND DOTY[8], after digesting the peptides according to the procedure of GRUNBAUM, SCHAFFER AND KIRK[9].

The radioactivity of the fractions from the Dowex columns was measured in aluminium cups, using Tracerlab Superscaler equipment with a TGC-2 end-window Geiger tube. Radioactive peptides were located on paper electrophoretograms and paper chromatograms by radioautography, using Ilford X-ray film.

Inactivation of enzyme with [^{32}P]DFP

The purified BAEE-hydrolase preparation was incubated at pH 7.5 and 20° with 40 μM [^{32}P]DFP (Radiochemical Centre, Amersham, England). Specific radioactivity: 50 μC/μmole). After 30 min, when most of the enzyme activity still remained, unlabelled DFP was added to a final concentration of 0.6 mM, to obtain complete inactivation. When the esterase activity had disappeared, a small sample of the incubation mixture was withdrawn for determination of the molar amount of phosphorus bound per mole of enzyme. The sample was chromatographed in water on a Sephadex G-50 column to remove excess [^{32}P]DFP from the protein. The eluted high-molecular weight material was analysed for phosphorus and protein content, and the molar ratio was calculated.

The main part of the inactivated enzyme was precipitated by the addition of

9 vol. of acetone. The precipitate was washed twice with acetone and dried *in vacuo*. The enzyme protein was hydrolysed with 12 M HCl (50 ml per g of enzyme) at 37° for 72 h (ref. 10). The hydrolysate was then brought to dryness several times in a rotary evaporator.

For comparison between the DFP-reacting sites of the three esterase components, enzyme preparations were incubated at pH 7.5 and 20° with [^{32}P]DFP (about $3.5 \cdot 10^6$ counts/min per μmole) at a concentration of approx. 1 mM. The inactivated enzymes were then precipitated with 0.25 vol. of 25% (w/v) trichloroacetic acid containing 0.01 M silicotungstic acid. The precipitates were washed 3 times with HCl–acetone (1 ml of conc. HCl in 250 ml of acetone), in order to remove unreacted DFP, and dried *in vacuo*. Hydrolysis was performed as described above.

Methods for isolation and analysis of ^{32}P-labelled phosphopeptides

Chromatography on Dowex 50. The acid hydrolysate of the [^{32}P]DFP-inactivated BAEE-hydrolase was chromatographed on a column of Dowex 50W-X8 eluted with 0.01 M HCl (ref. 11). The fractions were analysed for radioactivity and ninhydrin-reacting material.

Chromatography on Dowex 1. Pooled fractions from the Dowex 50 chromatography were chromatographed on columns of Dowex 1-X10 (Cl^-), which were eluted with HCl of gradually increasing concentration.

High-voltage paper electrophoresis. The pooled and dried fractions from the Dowex 1 chromatographies were analysed for purity, and in one case further purified by high-voltage electrophoresis on Whatman No. 3 paper in 1 M acetic acid (pH 2.3) using a Pherograph Original Frankfurt D.B.G.M., L. Hormuth, Heidelberg-Wiesloch, Germany. Electrophoresis was also performed at pH 3.5 and 5.0, in 0.05 and 0.1 M pyridine–acetic acid buffers, respectively.

Paper chromatography. The purity of labelled phosphopeptides was studied by paper chromatography, using the same systems as earlier[12].

Amino acid analyses. The major components from the Dowex 1 chromatography were hydrolysed in sealed tubes with 6 M HCl at 110° for 20 h, and brought to dryness in a vacuum desiccator over P_2O_5 and NaOH. Quantitative amino acid analyses were performed according to the method of SPACKMAN, STEIN AND MOORE[13]. A preliminary identification of the amino acids in the acid hydrolysates was made by high-voltage paper electrophoresis in 1 M acetic acid (pH 2.3).

Amino end-group analyses. The N-terminal residue of the peptides was identified by the method of SANGER[10]. The dinitrophenyl amino acids were separated by paper chromatography according to the method of BLACKBURN AND LOWTHER[15] with phthalate buffer (pH 6.0) and 10% ethanol–benzyl alcohol as solvents. The amino end-group analyses were checked by analysing for free amino acids in the acid hydrolysates of the dinitrophenyl phosphopeptides. This was performed by high-voltage paper electrophoresis at pH 2.3 with the appropriate amino acids as references.

RESULTS AND DISCUSSION

Isolation of labelled phosphopeptides from an acid hydrolysate of ^{32}P-labelled BAEE-hydrolase

Fig. 1 shows a Dowex 50 chromatogram of the acid hydrolysate from 1.2 g of

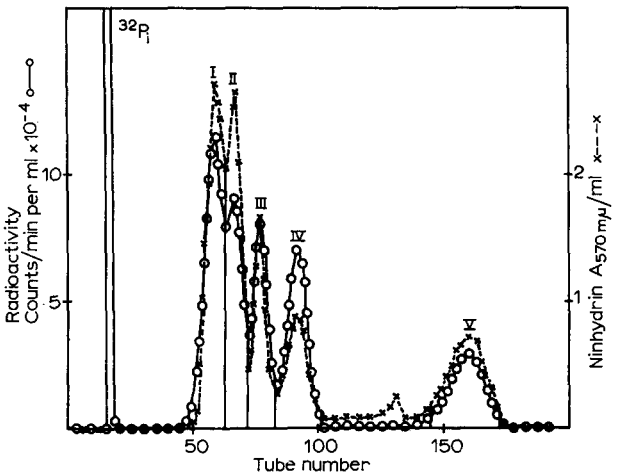

Fig. 1. Dowex 50W-X8 chromatogram of the acid hydrolysate of 1.2 g of [^{32}P]DFP-inactivated BAEE-hydrolase. Column dimensions: 3.0 cm × 30 cm. Elution performed with 0.01 M HCl. About 5 ml collected every 7.5 min. Fractions I through V pooled as indicated in the figure and chromatographed on Dowex 1-X10. ○—○, radioactivity, counts/min per ml; ×- - - -×, ninhydrin colour.

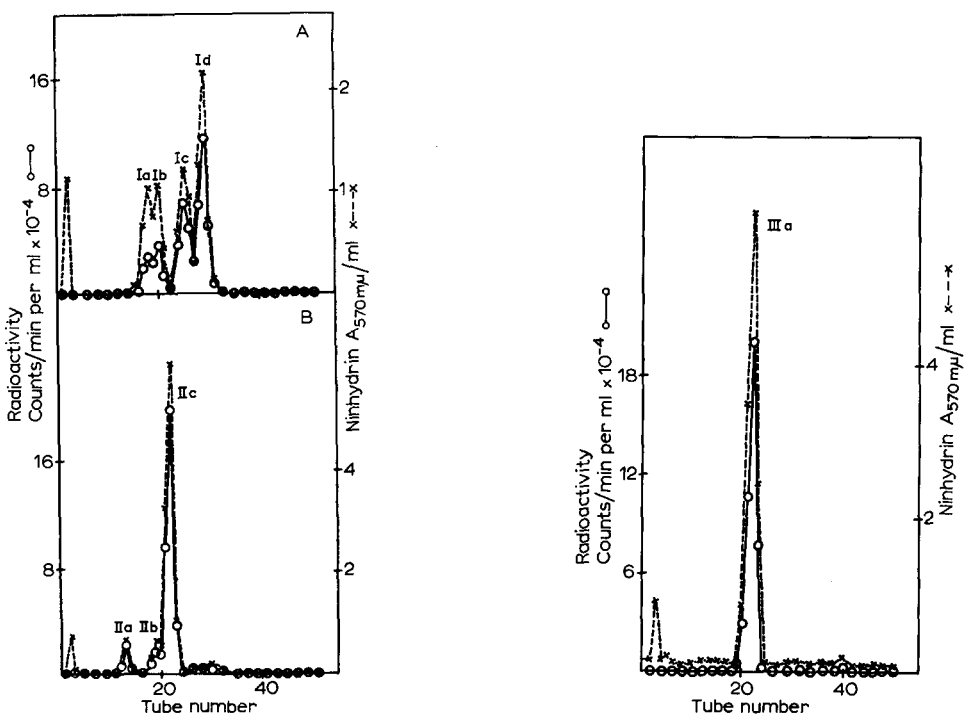

Fig. 2. Chromatogram of (A) Fraction I and (B) Fraction II from the chromatography in Fig. 1 on Dowex 1-X10 (Cl^-). Column dimensions: 1.3 cm × 19 cm. Elution with a linear gradient from 0 to 0.08 M HCl. Elution volume: 1 l. 5- to 6-ml fractions collected every 15 min. ○—○, radioactivity, counts/min per ml; ×- - - -×, ninhydrin colour.

Fig. 3. Chromatogram of Fraction III from the chromatography in Fig. 1 on Dowex 1-X10. Conditions and symbols identical with those in Fig. 2.

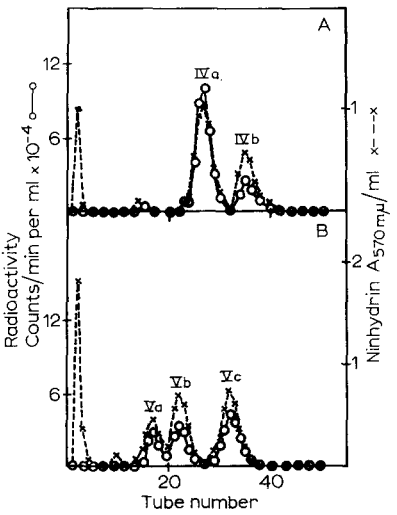

Fig. 4. Chromatogram of (A) Fraction IV and (B) Fraction V from the chromatography in Fig. 1 on Dowex 1-X10. Conditions and symbols identical with those in Fig. 2.

a [^{32}P]DFP-inactivated BAEE-hydrolase preparation. After the first radioactive peak, containing 17% of the radioactivity and identified as [^{32}P]orthophosphate, 5 main peaks with radioactive and ninhydrin-reacting material were eluted (Fractions I through V in Fig. 1). These fractions were pooled as indicated, dried and further chromatographed on Dowex 1 columns. The results are seen in Figs. 2–4.

Fraction I was resolved into 2 major (Ic and d) and 2 minor components (Ia and b) (Fig. 2A). Ic and d were homogeneous on high-voltage electrophoresis at pH 2.3, 3.5 and 5.0, as well as on paper chromatography.

Fraction II gave 1 major component (IIc) and 2 minor components (IIa and b) (Fig. 2B). IIc proved to be heterogeneous, and gave 2 bands on paper electrophoresis. It was, therefore, further fractionated at pH 2.3 by this method. The faster moving component (IIc 1) was found to run parallel to SerP. The other component (IIc 2) was eluted from the paper electropherogram and identified.

Fraction III was eluted from Dowex 1 as 1 peak (IIIa) with a constant radioactivity to ninhydrin ratio (Fig. 3). Further proof of its purity was obtained by electrophoresis and paper chromatography.

Fraction IV was resolved into 1 major (IVa) and 1 minor component (IVb) (Fig. 4A). On electrophoresis at pH 3.5, the former proved to contain 3 different components (IVa 1, 2, and 3 in order of decreasing mobility), of which IVa 3 was the major constituent. Furthermore, IVa 1 and 2 were found to migrate parallel to Id and IIc 2, respectively, on electrophoresis at pH 2.3, 3.5 and 5.0.

Fraction V, finally, was separated into 3 components (Fig. 4B) which, however, contained amounts of material too small for further examination.

Identification of major ^{32}P-labelled peptides

The amino acid composition, phosphorus content and N-terminal residues of the different peptides are given in Table I.

TABLE I

Amino acid composition and N-terminal residues of major phosphopeptides from [^{32}P]DFP-inactivated BAEE-hydrolase. Total amounts of phosphopeptides isolated from 1.2 g of enzyme were obtained from phosphorus determinations.

Component	*Total amount (μmoles)*	*Yield**	*Asp***	*Ser***	*Gly***	*N-terminal residue*	*Structure of peptide*
Ic	1.43	2.8	0.97	0.71	1.00	Asp	Asp–(SerP, Gly)
Id	2.45	4.8	0.95	0.76	0.00	Asp	Asp–SerP
IIc 2	1.03	2.0	0.96	0.72	0.00	Asp	Asp–SerP
IIIa	2.99	5.9	0.00	0.77	0.98	Ser	SerP–Gly
IVa	2.49	4.9	0.96	0.65	0.00	Asp	Asp–SerP

* Yield is defined as % of total phosphorus in phosphorylated enzyme.
** Expressed as moles of amino acids per mole of phosphorus.

Only the major components, *i.e.*, those containing at least 1 μmole of phosphorus per 1.2 g of starting material, were analysed.

The low serine values should reasonably be ascribed to destruction during acid hydrolysis, as has been shown to apply to peptides containing SerP (ref. 16), as well as to free SerP (ref. 17).

Components Id, IIc 2 and IVa contained only Asp and Ser, with Asp as the N-

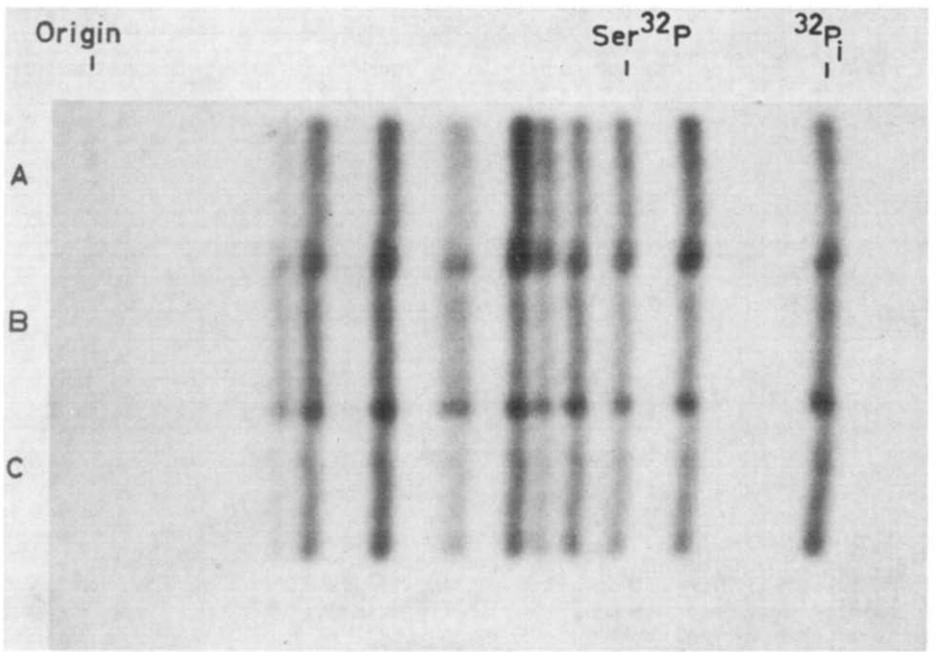

Fig. 5. Radioautograph of electrophoretogram of a partial acid hydrolysate of [^{32}P]DFP-inactivated Pronase esterases. (A) and (C) are components with PNPA-hydrolase activity and (B) is the component with BAEE-hydrolase activity. Amounts corresponding to 0.1 mg of each component were applied to a Whatman No. 3 paper on a continuous line at 5-cm intervals. Buffer: 0.05 M pyridine–acetic acid (pH 3.5). Voltage: 51 V/cm; electrophoresis time: 2 h, anodic migration. X-Ray film (Ilford, Ilfex) exposed for 8 days.

terminal residue. Consequently, it was concluded that Id (=IVa 1), IIc 2 (=IVa 2) and IVa 3 in all probability represented the three different Asp–SerP dipeptides described by NAUGHTON *et al.*[18], *i.e.*, β-Asp–SerP, α-Asp–SerP and α,β-Asp–SerP, respectively. Peptides Id and IVa 3 gave weakly coloured brownish spots after spraying the papers with the ninhydrin reagent and drying at about 100°. A similar reaction with ninhydrin has been described for the same Asp–SerP dipeptides from ovalbumin[12].

Asp–[^{32}P]SerP and [^{32}P]SerP–Gly were the only dipeptides identified. This implied that the tripeptide sequence Asp–Ser–Gly is part of the DFP-reacting site of the enzyme. This structure is further supported by the composition of Ic, whose amino acid sequence can be assumed to be Asp–[^{32}P]SerP–Gly. It should also be noted that rearrangements of amino acid sequences are negligible during hydrolysis with strong acid at low temperature[10].

As stated above, Pronase contains at least 2 additional DFP-sensitive components. Since these components have not been extensively purified, no investigation of their DFP-reacting site using the method described in this paper was made. Nevertheless, a preliminary investigation of the reactive site of these enzymes would be of interest. Consequently, it was shown by high-voltage paper electrophoresis at pH 3.5 that the same labelled phosphopeptides were obtained from the two PNPA hydrolases and the BAEE-hydrolase after incubation with [^{32}P]DFP and partial acid hydrolysis (Fig. 5). This indicates that all three esterases have the same primary structure around their reactive serine residues.

It has thus been demonstrated that the tripeptide sequence, including the DFP-reacting serine, of the *S. griseus* BAEE-hydrolase is Asp–Ser–Gly. Evidence has been obtained of the same sequence in the PNPA-hydrolases.

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