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THE PRIMARY STRUCTURE AT THE DFP-REACTING SITE OF A PROTEOLYTIC ENZYME FROM A STRAIN OF ARTHROBACTER

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SUMMARY

Two dipeptides, Ser–Ser^{32}P and Ser^{32}P–Gly, as well as a tripeptide containing Ser, Ser^{32}P and Gly with the serine as N-terminal residue, have been isolated from an acid hydrolysate of a proteolytic enzyme from a strain of Arthrobacter after inactivation with [^{32}P]DFP. This shows that the amino acid sequence around the reactive serine is Ser–Ser^{32}P–Gly, which has not been found hitherto in any DFP-sensitive enzyme.

INTRODUCTION

The purification of a proteolytic enzyme from a strain of Arthrobacter was recently described[1]. The enzyme has been shown to have esterase activity, and to be inactivated by DFP (ref. 2). During inactivation of the enzyme, a covalent link is formed between the phosphorus of DFP and a particular serine residue of the enzyme[3]. In addition, an acid hydrolysate of [^{32}P]DFP-inactivated enzyme has been shown to contain ^{32}P-labelled peptides which differed from those of bovine chymotrypsin treated in the same way, indicating a different amino acid sequence around the reactive serine in the two enzymes[3].

In this paper, an account is given of the isolation and identification of three ^{32}P-labelled peptides from the Arthrobacter protease. The amino acid sequence around the reactive serine residue is shown to be Ser–Ser^{32}P–Gly, a sequence not previously found in DFP-inactivated enzymes.

EXPERIMENTAL

Enzyme

The Arthrobacter enzyme was kindly supplied by Dr. B. VON HOFSTEN and Dr. U. BJARE, Institute of Biochemistry, Uppsala. Its activity was measured with the milk-clotting assay[4].

Analytical methods

Total phosphorus was determined as orthophosphate by the method of MARTIN

AND DOTY[5], after digestion according to the procedure of GRUNBAUM, SCHAFFER AND KIRK[6]. The radioactivity of the fractions from the Dowex columns was measured in aluminium cups, using a Sample Changer (L. K. B. Produkter AB, Stockholm, Sweden) fitted with a TGC-2 end-window Geiger tube, and connected to a Multi Matic Scaler and Auto Printer (Tracerlab, Waltham, Mass., U.S.A.).

Radioactive components in paper electropherograms were localized by radioautography, using Ilford X-ray film.

The position of peptides and amino acids were determined by spraying the paper electropherograms with the ninhydrin reagent of LEWIS[7].

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

200 mg of enzyme were dissolved in 20 ml of 0.05 M Tris–HCl buffer (pH 7.5) and dialysed overnight at 4° against the same buffer. It was then incubated at 20° with [^{32}P]DFP (Radiochemical Centre, Amersham, England) (about $2.5 \cdot 10^6$ counts/min per μmole) at a final concentration of $1.1 \cdot 10^{-3}$ M. After 4 h, when the enzyme was completely inactivated, the protein was precipitated by adding 1/4 vol. of 25% trichloroacetic acid–0.01 M silicotungstic acid. The precipitate was washed three times with HCl–acetone (1 ml conc. HCl in 250 ml of acetone) and dried *in vacuo*. Phosphorus analyses showed an incorporation of 0.83 mole of phosphorus per mole of enzyme.

Isolation and analysis of ^{32}P-labelled phosphopeptides

Hydrolysis of enzyme. The enzyme protein was hydrolysed with 12 M HCl at 37° for 72 h (ref. 8). The hydrolysate was then taken to dryness three times in a rotary evaporator.

Chromatography on Dowex 50. The acid hydrolysate of the [^{32}P]DFP-incubated enzyme was chromatographed on a Dowex 50W-X8 column with 0.01 M HCl as eluent[9]. The fractions were analysed for radioactivity.

Chromatography on Dowex 1. Pooled fractions from the Dowex 50 chromatography were further chromatographed on a Dowex 1-X10(Cl^-) column with gradually increasing concentration of HCl.

High-voltage paper electrophoresis. The purity of the pooled and dried fractions from the Dowex 1 chromatography was determined 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 pyridine–acetic acid buffers which were 0.05 and 0.1 M, respectively, with regard to pyridine.

Amino acid analyses. The radioactive components from the Dowex 1 chromatography were hydrolysed in sealed tubes with 6 M HCl at 110° for 20 h, and taken to dryness in a vacuum dessicator over P_2O_5 and NaOH. Quantitative amino acid analyses were performed according to SPACKMAN, STEIN AND MOORE[10].

Amino end-group analyses. The N-terminal residues of the isolated peptides were identified by the method of Sanger[11], with the modification that the DNP-peptides were hydrolyzed with 2 M HCl at 100° for 8 h, in order to protect DNP-SerP from complete hydrolysis. The DNP derivatives were identified by high-voltage paper electrophoresis in 1 M acetic acid (pH 2.3), together with the appropriate reference substances. Under these conditions, DNP-SerP, DNP-Ser and DNP-Gly separated. The position of ^{32}P-labelled DNP-SerP was also determined by radioautography.

DNP-SerP was prepared according to the general method for amino acids outlined by FRAENKEL-CONRAT, HARRIS AND LEVY[11]. The DNP-SerP was extracted from the acidified reaction mixture by ethyl acetate. DNP-Ser and DNP-Gly were purchased from Mann Research Laboratories.

RESULTS AND DISCUSSION

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

Fig. 1 shows a Dowex 50 chromatogram of the acid hydrolysate of 3 mg of [^{32}P]DFP-inactivated enzyme. After the first radioactive peak, which consisted of [^{32}P]orthophosphate, only two peaks with radioactive material appeared (Fractions I and II in Fig. 1).

Fraction I was homogeneous with respect to ninhydrin-reacting material and radioactivity on high-voltage electrophoresis at pH 2.3, 3.5 and 5.0. In these systems, it was identified as Ser^{32}P, using synthetic SerP (Calbiochem) as reference.

Fraction II proved to contain three components on electrophoresis at the aforementioned pH values, and was therefore further chromatographed on Dowex 1.

As seen in Fig. 2, Fraction II could be resolved into three peaks, IIa, IIb and IIc.

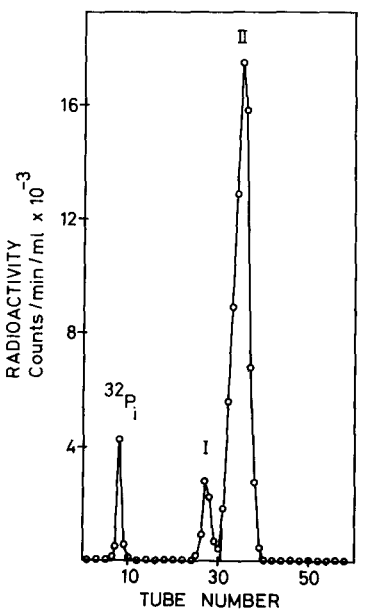

Fig. 1. Dowex 50W-X8 chromatogram of the acid hydrolysate of 3 mg of [^{32}P]DFP-inactivated Arthrobacter enzyme. Column dimensions: 1.2 cm × 45 cm. Elution performed with 0.01 M HCl. 3.0 ml collected every 12 min. Fraction II pooled as indicated in the figure, and chromatographed on Dowex 1-X10. ○—○, radioactivity (counts/min per ml × 10^{-3}).

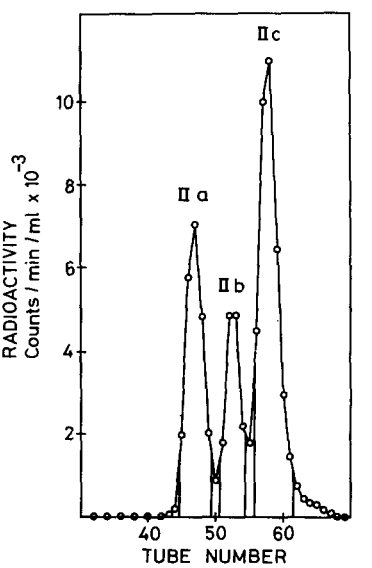

Fig. 2. Dowex 1-X10 chromatogram of Fraction II from the chromatography in Fig. 1. Column dimensions: 0.9 cm × 40 cm. Gradient elution with a linear gradient from 0 to 0.02 M HCl. Elution volume: 600 ml 2.3 ml. collected every 15 min. ○—○, radioactivity (counts/min per ml × 10^{-3}).

When pooled as indicated in the figure, the three components were found to be homogeneous on high-voltage electrophoresis at pH 2.3, 3.5 and 5.0.

Identification of ^{32}P-labelled peptides

The amino acid composition, phosphorus content and N-terminal amino acids of the different peptides purified by Dowex 1 chromatography are given in Table I.

TABLE I

AMINO ACID COMPOSITION AND N-TERMINAL RESIDUES OF PHOSPHOPEPTIDES FROM [^{32}P]DFP-INACTIVATED ARTHROBACTER PROTEASE

Total amounts of phosphopeptides isolated from 200 mg of enzyme were obtained from phosphorus determinations.

Component	*Total amount of phospho-peptide (μmoles)*	*Yield**	*Ser*	*Gly*	*N-terminal residue*	*Structure of peptide*
			(moles per mole of P)			
IIa	1.26	17.5	1.62	0.99	Ser	Ser–SerP–Gly
IIb	0.77	10.7	0.83	0.98	SerP	SerP–Gly
IIc	2.09	28.9	1.65	0.00	Ser	Ser–SerP

* Yield is defined as % of total phosphorus in phosphorylated enzyme.

The low serine values may be explained as due to destruction during acid hydrolysis, as has been shown to apply to peptides containing SerP (ref. 12), as well as to SerP itself[13]. Under the conditions used in this study, the amounts of serine were about 18% lower than would have been expected from the amounts of phosphorus and glycine.

The method of hydrolysis used for the DNP-peptides made it possible to distinguish between N-terminal Ser and SerP. The yield of DNP-SerP was approx. 35%, calculated from the radioactivity of the yellow spot after electrophoresis at pH 2.3. Besides DNP-SerP, some DNP-Ser was also formed. Furthermore, the amino end-group analyses were checked by analyzing for free amino acids in the acid hydrolysates of the DNP-phosphopeptides. This was performed with paper electrophoresis at pH 2.3, with the appropriate amino acids as reference substances.

Component IIb was further identified by its identical electrophoretic mobility with synthetic SerP-Gly (kindly supplied by Dr. G. FÖLSCH, Department of Medical Chemistry, University of Gothenburg, Sweden).

Taking into account the purity of the enzyme demonstrated by VON HOFSTEN, VAN KLEY AND EAKER[1], and the fact that 1 mole of enzyme reacts with 1 mole of DFP, resulting in a completely inactive enzyme[3], it can be concluded that each enzyme molecule contains one active site.

Consequently, since two of the peptides, IIb and IIc, were identified as SerP–Gly and Ser–SerP, the third peptide (IIa) must have the structure Ser–SerP–Gly.

Acid hydrolysis may, under certain conditions, cause a rearrangement of the amino acid sequence, as noted by SANGER[8]. The method used in this work (strong acid and low temperature) is, however, stated not to result in rearrangements to any measurable extent[8].

It can thus be concluded that the amino acid sequence around the reactive serine residue in the Arthrobacter enzyme is Ser–Ser–Gly, which differs from the active sites of other DFP-reacting enzymes hitherto reported[14].

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