crystallized once from ethanol. Yield 3.8 g = 49.7 %. M.p. 192 – 194 °C. (Found: C 77.5; H 5.6; N 3.8. Calc. for $C_{25}H_{21}NO_3$: C 78.3; H 5.5; N 3.7). Mass spectrum (rel. intensity): M+ 383, 279 (20.5), 278 (100), 105 (20.0), 91 (24.7), 77 (24.7).

*4,5-Diphenyl-2,3-b-pyrrolo-4,7-dimethylcoumarin* (III). Comarin I (1.9 g) and benzoin (2.2 g), or alternatively coumarin II (3.8 g), and *p*-TS (0.02 g) were mixed thoroughly and heated in an oil bath at 180 °C for 15 h. The crude product was treated with sodium hydrogen carbonate in water, dried and recrystallized twice from dimethylformamide (10 ml/g). Yield 0.7 g = 19.2 %. M.p. 323 – 325 °C. 0.2 g was chromatographed twice on preparative thin layer alumina with carbon tetrachloride-ethanol 25:1 for analysis. M.p. 330 – 331 °C. (Found C 82.3; 81.9; H 5.3; 5.0; N 3.7, 4.0. Calc. for $C_{25}H_{19}NO_2$: C 82.2; H 5.2; N 3.8). Mass spectrum M+ 365 (100), 337 (42.6), 323 (23.0).

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# *Pseudomonas* Cytochrome *c* Peroxidase. X. The Effect of *Pseudomonas* Neutral Proteinase on the Enzyme Molecule

RITVA SOININEN and NILS ELLFOLK

Department of Biochemistry, University of Helsinki, SF-00170 Helsinki 17, Finland

*Pseudomonas* cytochrome *c* peroxidase (PsCCP, EC 1.11.1.5) has been isolated in a homogeneous form as revealed by disc electrophoresis and analytical ultracentrifugation.[1,2] It consists of a single polypeptide chain with an apparent molecular weight of 40 000 ± 1 500 according to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS).[3] In some preparations, however, two smaller components were observed in SDS-gel electrophoresis. *Pseudomonas aeruginosa* was found to produce several proteolytic enzymes,[4] and therefore the smaller components were suspected to derive from PsCCP by proteolysis by a proteinase still present in the preparation.

In this study the effect of a partly purified *Pseudomonas* neutral proteinase on PsCCP was investigated.

*Experimental.* PsCCP was prepared as described previously.[1,2] The absorbance ratio $A_{407}/A_{280}$ of the preparations was 4.50 – 4.66. The purity of the preparations was checked by disc electrophoresis according to Maurer[5] (system No. 1a, pH 8.9, 7 % gel).

*Pseudomonas* neutral proteinase (elastase, "semialkaline proteinase") was prepared according to Morihara *et al.*[6] from a semisynthetic growth medium. After elution from DEAE-cellulose, the preparation was concentrated by ultrafiltration in a Diaflo apparatus (Amicon N.V., UM-10 membrane) and used without further treatment.

Incubation of PSCCP with the partly purified proteinase was carried out in 0.01 M Na-phosphate buffer pH 7.0 at 40 °C at an enzyme-substrate ratio appr. 1:100 (w/w). The reaction was stopped by inactivating proteinase by heating at 100 °C for 5 min.

Analytical SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn[7] in 10 % gels. Standard proteins used for molecular weight estimation were: bovine serumin albumin (Fraction V, Armour), ovalbumin (Grade V, Sigma), pepsin (crysallized, Sigma), and horse heart cytochrome *c* (Type III, Sigma). Analytical SDS-polyacrylamide gel electrophoresis in 12.5 % gels in the presence of 8 M urea according to Swank and Munkres[8] was used to separate the peptides in the 1 500 to 10 000 molecular weight range. 10 – 30 μg of protein were applied per gel. After electrophoresis the gels were cut in the middle of the marker dye band, stained with

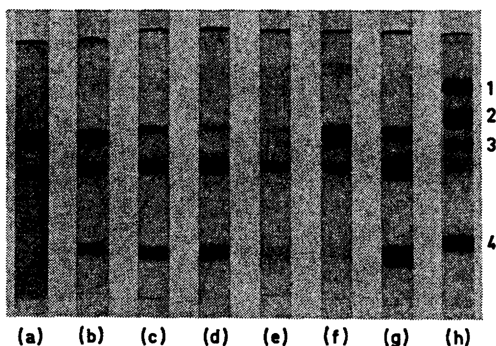


*Fig. 1.* SDS-polyacrylamide gel electrophoresis in 10 % gels according to Weber and Osborn [7] of untreated PsCCP (a); of PsCCP incubated with *Pseudomonas* neutral proteinase for 1 (b), 5 (c), and 30 (e) min in 0.01 M Na-phosphate buffer pH 7.0 at 40 °C; of PsCCP incubated without proteinase for 30 min under the same conditions as before (f); of a PsCCP preparation cleaved during the purification (g); and of standard proteins (h): 1, serum albumin; 2, ovalbumin; 3, pepsin; 4, horse heart cytochrome *c*.

Coomassie Brilliant Blue R 250 (Serva) and de-stained by soaking with anion exchange resin (Weber *et al.*).[9] The gels were photographed as described by Oliver and Chalkley.[10] SDS (pract., Fluka) and urea (pure, Merck) were recrystallized before use.

Preparative SDS-polyacrylamide gel electrophoresis in a 10 % gel was performed on a horizontal gel bed (100 × 150 × 3 mm). With slight modifications the procedure used was that of Weiner *et al.*[11] The gel was cast in a rectangular mold with a groove (2 × 85 × 2 mm) for sample application 2 cm from one end of the gel bed. After polymerisation the gel was pre-eluted by soaking for 4 days at 30 °C with gentle agitation in the electrophoresis buffer, which was changed every 8 – 16 h. β-Mercaptoethanol was omitted from the sample. 3 mg of protein was applied to the gel, and the electrophoresis was run in a Desaga thin-layer electrophoresis apparatus at 60 mA for 16 h at 25 °C. After this time a guide strip was cut from the edge of the gel and the rest of the gel was frozen. The guide strip was stained and de-stained in the same way as the analytical gels. Zones containing polypeptides were cut off, and eluted twice with 5 mM $NaCO_3$ containing 0.05 % SDS for 8 – 16 h at 30 °C. The eluate was centrifuged and lyophilised.

*N*-Terminal amino acids and *N*-terminal amino acid sequences were determined by the dansyl-Edman procedure in the presence of SDS.[11]

The protein concentration of PsCCP was determined using the absorption coefficient at 280 nm $A$(1 cm, 1 %) = 12.1,[12] and that of the proteinase preparation by the Folin-Ciocalteau reaction.[13]

Chemicals were of analytical grade if not otherwise stated.

*Results.* The electrophoretic patterns of the samples from the incubation mixture of PsCCP and the partly purified *Pseudomonas* neutral proteinase are shown in Fig. 1 (b – e). Two polypeptide bands with apparent molecular weights of about 28 000 and 11 000, as calculated from the relative mobilities in SDS-gels, appear during the incubation. No peptides with lower molecular weights than 11 000 were observed in SDS-gel electrophoresis in the presence of 8 M urea within the first 30 min incubation time (Fig. 2). After prolonged incubation (60 min), smaller peptides appeared and larger peptides disappeared completely. Fig. 1 (f) and Fig. 2 (f) show the electrophoretic patterns of PsCCP incubated for 30 min without the addition of proteinase. Two faint bands appeared indicating the presence of some contaminating proteinase. Fig. 1 (g) and Fig. 2 (g) show the electrophoretic patterns of a PsCCP preparation suspected to be cleaved during the purification procedure. In addition to the band corresponding to the intact PsCCP, two faster moving bands were present. These had the same apparent molecular weights as those obtained by the cleavage of PsCCP by the added proteinase.

In order to study further the cleavage products, PsCCP was treated with proteinase for 10 min on a larger scale under the same conditions as before, the polypeptides being isolated by preparative SDS-gel electrophoresis (Fig. 3A, Preparations A-b and A-c). The polypeptides from the PsCCP preparation cleaved during the purification were isolated by the same method (Fig. 3B, Preparations B-b and B-c). The *N*-terminal amino acid sequence of Preparations A-c and B-c was the

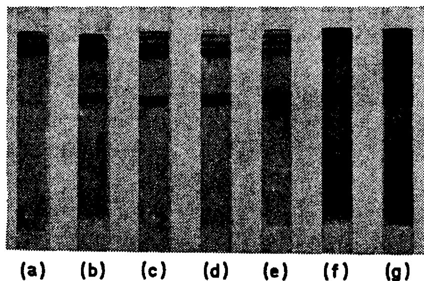


*Fig. 2.* SDS-polyacryalmide gel electrophoresis in 12.5 % gels in the presence of 8 M urea according to Swank and Munkres [8] of untreated PsCCP (a); of PsCCP incubated with and without *Pseudomonas* neutral proteinase as described in Fig. 1 (b-f); and of a PsCCP preparation cleaved during the purification (g).

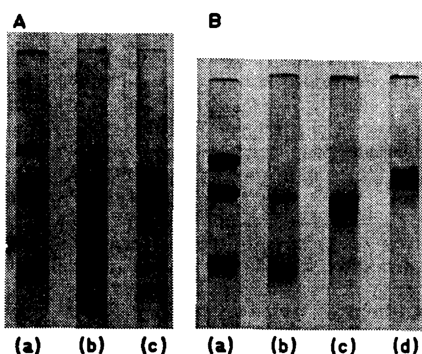


*Fig. 3.* Isolation of the polypeptide fragments obtained (A) by the limited proteolysis of PsCCP by *Pseudomonas* neutral proteinase (incubation of PsCCP with proteinase, appr. 100:1 w/w, in 0.01 M Na-phosphate buffer pH 7.0 for 10 min at 40 °C) and (B) from PsCCP cleaved during the purification. The preparations were analysed by analytical SDS-gel electrophoresis according to Weber and Osborn [7] in 10 % gels prior (a) and after (b-d) the preparative SDS-gel electrophoresis.

same as that of the intact protein: *Asp-Ala-Leu-*. Presumably these fractions arose from the *N*-terminal part of the protein molecule. More than one *N*-terminal amino acid was found in Preparations A-b and B-b that gave the apparently homogeneous bands of molecular weight 11 000 in the electrophoresis. The *N*-terminal amino acids in Preparation A-b were leucine, phenylalanine and valine, while those in Preparation B-b were leucine, phenylalanine and, obviously arising from the larger polypeptide fraction seen faintly in Fig. 3B, b, traces of aspartic acid. This indicates that the faster moving bands, and, correspondingly, the slower ones as well, consist of 2 – 3 fragment of about the same molecular weight. The *N*-terminal amino acids of the faster moving fragments were those expected to arise due to the hydrolysis catalysed by neutral proteinase, which cleaves the peptide bonds from the imino side of hydrophobic amino acids.[14]

*Discussion. Pseudomonas* neutral proteinase cleaves PsCCP into two large fragments similar to those arising in some purified preparations. The molecular weights of PsCCP and neutral proteinase, and the isoelectric points as well, are of the same magnitude.[1,6,12] Therefore it may be difficult to separate the two proteins completely by the methods used in the purification procedure for PsCCP (ion exchange and gel chromatography). PsCCP can, however, be prepared without noticeable proteolytic cleavage when all steps are carried out at a low temperature without unnecessary delay. The presence of the cleavage products in the final PsCCP preparation can easily be checked by SDS-gel electrophoresis as described in this paper.

The cleavage of the PsCCP molecule into a few, large, well-defined fragments, together with the *N*-terminal amino acid analyses of these, indicates that a few peptide bonds in the polypeptide chain are preferentially attacked by neutral proteinase.

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