Short Communications

The Trypsin-Like Enzyme from *Streptomyces griseus* (Pronase)

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(Received 24 April 1968)

The presence, in Pronase, of a trypsin-like activity that hydrolyses BAEE* and that, with BAEE or casein as substrate, is inhibited by the soya-bean trypsin and α -chymotrypsin inhibitor AA (Birk, 1961; Birk, Gertler & Khalef, 1963), has been reported (Nomoto, Narahashi & Murakami, 1960; Wählby, Zetterqvist & Engström, 1965; Birk, 1966). Isolation and study of this trypsin-like activity and its comparison with mammalian trypsin was then attempted.

Commercial Pronase (B grade, lot 54909; Calbiochem, Los Angeles, Calif., U.S.A.) was fractionated on CM-cellulose. All operations were performed at 4°. A sample (50 mg.) was suspended in 5 ml. of 0.01 M-ammonium acetate buffer, pH 4.6 (starting buffer), and then centrifuged for 20 min. at 15 000 rev./min. (27 000 g). The clear supernatant was applied to a CM-cellulose column (1 cm. $\times$ 13 cm.) previously equilibrated with the starting buffer. Elution was performed by the starting buffer until the E_{280} value of the eluate began to return to the base-line value. A gradient with respect to buffer concentration and pH was then started by introducing 0.1 M-ammonium acetate buffer, pH 6.9, through a 100 ml. mixing chamber containing the starting buffer. The gradient was increased by substituting 0.2 M-ammonium acetate buffer, pH 6.9, as the solution flowing into the mixing chamber. The flow rate was adjusted to 60 ml./hr. and 3 ml. fractions were collected. Every second tube was assayed for protein content by determining E_{280} and every second to fourth tube for esterolytic activity on BAEE and on ATEE by the method of Schwert & Takenaka (1955), for proteolytic activity on casein by the casein-digestion method of Kunitz (1947) and for the extent of inhibition by trypsin inhibitors as described by Birk *et al.* (1963). BAEE and ATEE were products of Mann Research Laboratories Inc. (New York, N.Y., U.S.A.). Vitamin-free casein was purchased from Nutritional Biochemicals Corp.

* Abbreviations: BAEE, N^{α} -benzoyl-L-arginine ethyl ester; ATEE, N -acetyltyrosine ethyl ester.

(Cleveland, Ohio, U.S.A.) and inhibitor AA was prepared by the method of Birk & Gertler (1968). A typical separation pattern of the protein and of the activities is given in Fig. 1. Fractions A₂, B, C and D possess proteolytic activity, but only fraction C and to a very small extent fraction D hydrolyse BAEE and are inhibited by inhibitor AA and by other trypsin inhibitors such as Kunitz's crystalline soya-bean trypsin inhibitor, the pancreatic trypsin inhibitor (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), and the lima-bean and egg-white trypsin inhibitors (Nutritional Biochemicals Corp.). Although the Pronase solution was slightly active on ATEE before fractionation, this activity could not be detected in any of the effluent fractions from the column. Rechromatography of fraction C, which possesses the bulk of trypsin-like activity (contents of tubes 85-97; Fig. 1a), was performed on a CM-cellulose column (0.75 cm. $\times$ 10 cm.) equilibrated as described above. The effluent in tubes 85-97 from the first column was combined and delayed against the starting buffer, and a sample corresponding to 2.5 mg. of protein was applied to the column. Elution was performed as described in Fig. 1(b) and further purification of fraction C from accompanying residues of A, B and D was thus achieved. Rechromatographed fraction C was stable in the cold as a freeze-dried preparation.

The purity of the final trypsin-like enzyme (CM-cellulose effluent fraction C) is shown by its constant specific activities on BAEE and casein and by its extent of inhibition by inhibitor AA throughout the peak (Fig. 1b). This was further substantiated by electrophoresis in polyacrylamide gel at pH 4.5 (Reisfeld, Lewis & Williams, 1962). A sample (300 μ g.) of fraction C, applied in 40% (w/v) sucrose, migrated as a single band. Fraction C also emerged as a single peak on exclusion chromatography on Sephadex G-25, G-75 and G-100 columns, and with the hold-up volume on chromatography on DEAE-cellulose equilibrated with 7.5 mM-ammonium acetate buffer, pH 8.5.

Fig. 1. (a) Chromatographic separation pattern of Pronase (50 mg. in 5 ml. of 0.01 M-ammonium acetate, pH 4.6, starting buffer) on a CM-cellulose column (1 cm. $\times$ 13 cm.): (1) starting buffer, total volume of eluting agent 150 ml.; (2) gradient, with respect to buffer concentration and pH, formed by introducing 0.1 M-ammonium acetate, pH 6.9, through a 100 ml. mixing chamber containing the starting buffer, total volume of eluting agent 90 ml.; (3) increased gradient formed by substituting 0.2 M-ammonium acetate, pH 6.9, as the solution flowing into the mixing chamber, total volume of eluting agent 210 ml. The flow rate was 60 ml./hr. with effluent collected in 3 ml. fractions. (b) Rechromatography of a sample, corresponding to 2.5 mg. of protein, from the dialysis residue of fraction C (tubes 85–97, Fig. 1a) on a CM-cellulose column (0.75 cm. $\times$ 10 cm.): (1) starting buffer, total volume of eluting agent 45 ml.; (2') gradient, with respect to buffer concentration and pH, formed by introducing 0.08 M-ammonium acetate, pH 6.9 (60 ml.), through an 80 ml. mixing chamber containing the starting buffer, total volume of eluting agent 60 ml.; (3') increased gradient formed by substituting 0.16 M-ammonium acetate, pH 6.9 (60 ml.), as the solution flowing into the mixing chamber, total volume of eluting agent 60 ml.; (4) further increased gradient formed by substituting 0.24 M-ammonium acetate, pH 6.9, as the solution flowing into the mixing chamber, total volume of eluting agent 45 ml. The flow rate was 60 ml./hr. with effluent collected in 3 ml. fractions. —, Protein content expressed as E_{280} ; O, activity on BAEE, expressed in E_{253} (determined by the method of Schwert & Takenaka, 1955); ●, extent of inhibition by inhibitor AA, expressed in E_{280} (determined by the method of Birk *et al.* 1963); Δ, activity on casein, expressed in E_{280} (determined by the method of Kunitz, 1947).

The complex that the present trypsin-like enzyme forms with inhibitor AA (Birk, 1966) was dissociated when submitted to paper electrophoresis in 0.2 M-pyridine-acetate buffer, pH 4.9, for 8 hr. in the cold, whereupon the enzyme and inhibitor resume their original activities.

As to other proteolytically active fractions from the column, fractions A₂ and D showed only slight contamination when submitted to electrophoresis in polyacrylamide gels. Their specificity has not yet been established.

Since Pronase, as a whole, appeared to be homogeneous in the ultracentrifuge, no ultracentrifugal analyses of the individual fractions were attempted.

These results clearly indicate that fraction C isolated from Pronase is a trypsin-like enzyme.

The authors are grateful to Mrs S. Khalef for her valuable assistance.

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