A New Alkaline Protease from *Acremonium kiliense*

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This paper describes the preparation and purification of a new proteolytic enzyme isolated from the fungus *Acremonium kiliense* Grütz (which has often been called *Cephalosporium acremonium*). The enzyme has a pH optimum of 10.5, and its biosynthesis is stimulated by Zn^{2+} (properties that distinguish it from the crude enzyme isolated from an unidentified *Cephalosporium* by Oleniacz & Pisano, 1968). Pisano *et al.* (1963) reported protease production by several species of *Cephalosporium*, including '*Cephalosporium acremonium*', but the enzymes produced were not characterized in detail and it is impossible to tell if their enzyme is the same as this one.

A crude preparation of the enzyme was specially manufactured by Marchon Italiana S.p.A., Castiglione delle Stiviere (Mantova), Italy, to whom we are very grateful for a generous supply. The organism was grown by the manufacturers in an enriched medium containing Zn^{2+} . After several days of growth the medium was filtered and concentrated by evaporation to produce a fine brown powder, which was dried under reduced pressure. The proteolytic activity of this powder and of the enzyme purified from it was assayed by measuring the extinction at 280nm of trichloroacetic acid-soluble material released by the enzyme from a solution (5mg/ml) of casein in 0.1M-boric acid adjusted to pH10.5 with 1M-sodium hydroxide (see Kunitz, 1947).

Purification of the enzyme from the crude powder was accomplished essentially with one chromatographic column only. In a typical small-scale experiment 5g of crude material was dissolved in 100ml of water and the solution was centrifuged to remove insoluble material (less than 1% of the total). The solution was then dialysed twice at 4°C against 2 litres of acetate buffer (30mM with respect to sodium acetate and 30mM with respect to acetic acid, pH about 4.7). This step is essential to remove from the crude solution small

molecules that would interfere with column chromatography: the pH and conductivity of the solution after dialysis must be exactly the same as those of the buffer.

A column (1cm×19cm) of CM-cellulose (Whatman CM-52, microgranular) was equilibrated with the same buffer, and the dialysed protein solution passed through it at about 60ml/h. The column was washed with more buffer until the extinction of the effluent at 280nm became negligible. During this operation most of the brown colour of the crude material passed straight through the column, but all of the enzymic activity remained bound to the CM-cellulose.

The column was then developed with acetate buffer (60mM with respect to sodium acetate and 60mM with respect to acetic acid, i.e. twice the previous concentration but the same pH). Fig. 1 shows that this buffer immediately elutes a little 280nm-absorbing material, but that active enzyme is not eluted until after the column has become equilibrated with the more concentrated buffer (as shown by the conductivity of the effluent solution). This means that the enzyme has been moving on the column under conditions of true equilibrium chromatography, which is a more sensitive method of purification than those that depend on elution by a moving boundary of changed buffer.

The eluted enzyme was concentrated to about 10ml by ultrafiltration (in an Amicon model 12 cell with a UM-10 membrane operating under N_2 at 2bar pressure), dialysed against 1M-hydrochloric acid (2 litres) and freeze-dried. Activity is lost on freeze-drying if the enzyme concentration is less than about 1mg/ml. The yield is about 10mg of enzyme from 5g of starting material, which is a 70% recovery of proteolytic activity.

The purification can be carried out on a larger scale either in the same way or by making a solution of the crude enzyme so dilute that the concentration of salts is low enough for it to be chromatographed directly. In this case it is necessary to use fibrous CM-cellulose (Whatman CM-23) to obtain fast enough flow rates, and it is usually necessary to

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Fig. 1. Chromatography on CM-cellulose of a proteolytic enzyme from *Acremonium kiliense*. The histogram shows the extinction of the eluate at 280 nm (1 cm path length). ○, Conductivity of the eluate; the conductivity of the more dilute buffer was $1.3 \text{ m} \cdot \Omega^{-1}$, and that of the more concentrated buffer was $2.9 \text{ m} \cdot \Omega^{-1}$. ●, Enzyme activity (as extinction at 280 nm of a trichloroacetic acid supernatant; see the text).

repeat the purification with microgranular cellulose.

The final product was homogeneous on electrophoresis in polyacrylamide gels at pH 4.3 and 9.5 and in the presence of sodium dodecyl sulphate (at a concentration of 0.1%; Weber & Osborn, 1969). [The protein stains weakly on these gels: over $75 \mu\text{g}$ is needed to produce a visible stain with Coomassie Brilliant Blue R (Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.).] The protein was also homogeneous as judged by sedimentation-equilibrium studies in the ultracentrifuge. Although the purification procedure is a very simple one, these criteria do suggest strongly that the enzyme is pure.

Gel-filtration chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden) in 1M-hydrochloric acid and on Sephadex G-100 in 50mM-tris-HCl buffer, pH 7.5, containing 0.1M-potassium chloride showed a molecular weight of about 28000 with no sign of active aggregates. Electrophoresis in the presence of sodium dodecyl sulphate gave a similar value.

The enzyme has several properties that distinguish it from other proteases from moulds and bacteria. The optimum pH for digestion of casein was found to be about 10.5 in phosphate, borate or bicarbonate buffers. It remains active over a wide range of pH (down to below pH 5), but activity is rapidly lost at pH values above 12.5 because of irreversible denaturation. Like many proteolytic

enzymes it is stable at low pH and can be stored at 4°C and at a concentration of 1 mg/ml for many weeks at pH values between 3 and 7 without loss of activity. This stability is not affected by 8M-urea, in which the enzyme is fully active even after it has been incubated in 8M-urea solution overnight at room temperature. Although several other highly alkaline proteases have been found in bacteria and moulds (e.g. *Aspergillus oryzae*; Nordwig & Jahn, 1968), these have pH optima significantly lower than 10.5. The stability towards 8M-urea is uncommon among enzymes, but a few other examples have been reported (e.g. thermolysin; Ohta, 1967).

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