ATPase may be obtained by plotting $1/[Na^+]$ against $\sqrt[n]{-1/v}$, where the numerical value of n giving a linear relationship is equivalent to the number of Na^+ required to activate the enzyme. Fig. 2 shows that a straight line is obtained when n equals 2 but not when n equals 1. This result suggests that two Na^+ and one K^+ are required to activate the enzyme, a result similar to that seen with brain[6]. The fact that maximum activity occurs at a Na^+/K^+ ratio of 16 and it requires only two Na^+ to one K^+ to activate the enzyme suggests that K^+ has a much higher affinity for the active site of the enzyme.

The inhibition of ATPase activity by higher concentration of Na^+ was further investigated over the range 12–72 mM. The results obtained, plotted by the method of DIXON[7], are shown in Fig. 3. The linear relationship obtained indicates that the site of inhibition by Na^+ is probably the same as that involved in the activation by K^+.

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Activity against a synthetic substrate by a preparation of extracellular proteinase from *Serratia marcescents*

Preparations of extracellular proteinase from several strains of the bacterium *Serratia marcescens* have been found to digest a wide range of proteins and with these substrates showed optimal activity at about pH 9 (refs. 1–5). Purified proteinase was found to split carboxymethylated B chain of insulin at 22 bonds and peptides containing not less than 3 residues were formed[4]. The biological activity of kinin-9 (a nonapeptide) was destroyed by the enzyme[5] but benzoyl arginine ethyl ester[4], benzoyl tyrosine ethyl ester[1], peptides ranging from diglycine to hexaglycine[4], polylysine[2] and polyaspartic acid[2] were unhydrolysed. In the present work proteinase isolated from *S. marcescens* NCIB 10351 proved able to hydrolyse Z–Gly–Pro–Gly–Gly–Pro–Ala at the Gly–Gly bond. This observation could facilitate characterisation of the enzyme.

For production of proteinase, cultures were grown at 28° with aeration in 400-ml volumes of 2% peptone, 0.2% disodium phosphate containing salts of trace metals including calcium. They were started with 50-ml cultures initiated with loop inocula and were incubated for 24 h. The pH of the medium changed from 6.9 to 8.6. Cultures were centrifuged at 4°, the supernatant was adjusted to pH 6.7 with acetic acid, $CaCl_2$ was added (1 ml of 1 M $CaCl_2$). $(NH_4)_2SO_4$ was added to a final concentration of 40% and the solution was allowed to stand for 24 h at 4°. The precipitate was collected by centrifugation and dissolved in pH 6.7 buffer containing 0.1 M NaCl. (Throughout this work buffer solutions were at pH 6.7, contained 1 mM (*N*-morpholino)ethanesulphonic acid[6], 1 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid[6] (Calbiochem), 1 mM Tris, and unless otherwise stated 1 mM $CaCl_2$.) The solution of crude enzyme was dialysed against this buffer containing 0.1 M NaCl, and red pigment was removed by adsorption on a short column of ECTEOLA-cellulose equilibrated with a similar NaCl-buffer solution. Effluent containing proteinase (assayed with casein[7]) was dialysed against 0.1 mM $CaCl_2$ and freeze dried, and most of the non-proteolytic protein was removed by gel filtration through Sephadex G-75. Proteinase activity was found in a single peak; the active fractions were pooled, dialysed as above and freeze dried. This preparation was used for the preliminary characterisation of the proteinase described later.

Further purification was achieved by ion-exchange chromatography on ECTEOLA-cellulose in pH 6.7 buffer using a linear gradient of NaCl (0–0.1 M). Some non-proteolytic material passed through the column immediately, and on application of the salt gradient, several minor inactive components emerged, followed by proteinase which comprised most of the protein. Although during the elution of the proteinase the absorption at 280 nm and the activities against casein and the hexapeptide substrate were parallel, the profile of the active peak was always asymmetric, suggesting the presence of more than one component with activity against both substrates and eluting from the column in close succession. All active fractions from this peak were pooled, dialysed and freeze dried.

This pooled material was examined by isoelectric focusing in polyacrylamide gel[8] using Ampholine (LKB, pH range 3–10), and tested for activity against casein[9] at pH 9. Gels were stained with Coomassie Blue in 10% trichloroacetic acid following exhaustive extraction of the Ampholine from the gel with 10% trichloroacetic acid; the protein bands reached maximal intensity of staining after about 48 h. Several proteolytic bands focusing around pH 5 were observed but no non-proteolytic protein could be detected.

An estimate of the molecular weight of the active fraction isolated by ion-exchange chromatography was obtained by gel filtration[10] with Sephadex G-75 and using bovine pancreas ribonuclease (Sigma, chromatographed grade Type II), whale myoglobin (Seravac), bovine β-lactoglobulin (Pentex) and ovalbumin (Pentex 5 times crystallised) as standards. Protein and enzyme active against casein ran as a single peak with an elution volume corresponding to a molecular weight of 39 000. This differs from the previously reported values: 20 000 (ref. 2), 30 000 (ref. 3) and 67 000 (ref. 1).

The proteinase fraction obtained by gel filtration on Sephadex G-75 was inactive against Leu–Gly–Gly, Z–Gly–Tyr, Z–Gly–Phe and Z–Gly–Leu–NH_2, and only slightly active against benzoylarginine naphthylamide. However, this preparation hydrolysed Z–Gly–Pro–Gly–Gly–Pro–Ala to Z–Gly–Pro–Gly and Gly–Pro–Ala. *Clostridium histo-*

lyticum collagenase, used as control, gives the same products[11]. Another substrate for histolyticum collagenase, P–Pro–Leu–Gly–Pro–D-Arg[12], was not hydrolysed. The digestion products were identified by thin-layer chromatography on silica gel[13] using Eastman Chromogram Sheet 6060. The enzyme released ninhydrin-positive material from bovine tendon (Worthington), and hydrolysed the B chain of *S*-carboxymethyl-insulin. The products from the digestion of the insulin chain were examined by the dansyl end group method[14]. In accord with previously reported data[4], a number of bonds were split and no distinctive specificity pattern was discernible.

The enzyme showed optimal activity at pH 9 with casein or tendon as substrates and an optimum at pH 5 with the synthetic substrate. As judged by activity against casein the proteinase was most stable at 4°. The enzyme lost activity slowly at 4° when dialysed against pH 6.7 buffer not containing added metal salts or containing 0.1 mM EDTA. After dialysis to remove metal salts the enzyme had a half life at 35° of about 10 h and was rapidly inactivated with EDTA. In the absence of EDTA, NaCl (0.1 M) or 1 mM $CaCl_2$ decreased the rate of inactivation at 35° but 1 mM Co^{2+}, 1 mM Mn^{2+} or 1 mM Mg^{2+} had no effect. Inactivation by EDTA was not reversed by 1 mM excess of the above metal ions. Zn^{2+} (0.1 mM) rapidly inactivated the proteinase. Activity against casein and the synthetic substrate was not lowered by incubation with 1 mM diisopropylfluorophosphate in carbonate buffer at pH 8.2.

Although as far as possible the enzyme was processed at 4°, chromatography was carried out at 20°, and some autodigestion might have occurred. The formation of smaller but still active components by autolysis may in part account for the chromatographic and electrofocusing patterns obtained but the presence of isoenzymes or differing enzymic species as reported for the collagenase of *C. histolyticum*[15] cannot yet be ruled out.

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