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PREPARATION OF PORCINE ENTEROPEPTIDASE FREE OF INTESTINAL AMINOPEPTIDASE ACTIVITY WITH CON A-SEPHAROSE

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Summary

Chromatography on Con A-Sepharose is shown to be a rapid and efficient method for the preparation of porcine enteropeptidase free of intestinal aminopeptidase (EC 3.4.21.9) activity. This method is to be preferred because of its selectivity and simplicity.

The preparation of enteropeptidase (EC 3.4.21.9) from pig duodenal fluid had proved difficult since enzyme samples were always contaminated with aminopeptidase activity [1,2]. Baratti et al. [2] reported a separation of these two activities on a Sephadex G-200 column with a void volume of 4 l. However in our hands this separation was always incomplete and the isolation of enteropeptidase free of the aminopeptidase was only achieved at the expense of excessive losses of enteropeptidase activity especially when handling small protein loads. Therefore a more suitable means of separating these two intestinal enzymes was sought. Because of the high carbohydrate content reported for porcine enteropeptidase (about 37%) [2] a separation on the basis of the carbohydrate component was attempted using Con A-Sepharose. This support successfully brought about a rapid, complete and reproducible separation of the two activities with good recovery of the enteropeptidase activity thereby avoiding the tedious preparation of antisera against kidney aminopeptidase and subsequent immunoadsorption of the intestinal aminopeptidase activity [3].

Enteropeptidase activity was determined as previously described [4] using bovine trypsinogen as substrate but with one modification. After the trypsinogen activation had been stopped by the addition of 0.55 M HCl the assay mixtures were incubated at 50°C for 10 min. This step ensured complete

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inactivation of the enteropeptidase, traces of which may otherwise be carried over to the trypsin determination causing an exponential release of *p*-nitroaniline from the substrate benzoyl arginine *p*-nitroanilide. No trypsin was destroyed during the incubation. Aminopeptidase activity was determined spectrophotometrically using a method modified from that of Roncari and Zuber [5]. Assay mixtures (1 ml) contained 0.05 M phosphate (K^+), pH 7.2, and 1 mM leucine *p*-nitroaniline (Cyclo Chemical Co.). Incubation was at 30°C and the increase in absorbance at 410 nm was recorded following the addition of 20-μl aliquots of enzyme. One unit of aminopeptides activity hydrolysed 1 μmol of substrate per min.

Enteropeptidase was prepared from either duodenal fluid or duodenal mucosa. An acetone powder was prepared from fresh pig duodenal fluid and the powder was extracted with 0.01 M acetate (Na^+), pH 5.0. On the other hand the enzyme was extracted from freeze-dried pig duodenal mucosa by a method modified from that of Baratti et al. [2] using 1% (v/v) Triton X-100 instead of sodium deoxycholate. In each case the extract was fractionated on DEAE-Cellulose (Whatman DE 23), pH 6.0, with a salt gradient to 0.35 M NaCl and further fractionation was achieved by gel-filtration on Sephadex G-200, pH 6.0, followed by ion-exchange chromatography on DEAE-Sephadex (A-50), pH 8.0, with a salt gradient to 0.25 M NaCl in 0.01 M Tris (Cl^-), pH 8.0. Sephadex G-200 gel-filtration of these enzyme preparations showed poor resolution of the enteropeptidase and aminopeptidase activities and this was not greatly improved following two recycles through the same column. Fig. 1 shows the elution profile of enteropeptidase prepared from duodenal fluid, while preparations from duodenal mucosa also showed this same poor resolution of these two activities.

Enteropeptidase was separated from the intestinal aminopeptidase on Con A-Sepharose (Pharmacia Fine Chemicals) using a method modified from that of Lloyd [6]. The enzyme was equilibrated with 0.01 M Tris (Cl^-), pH

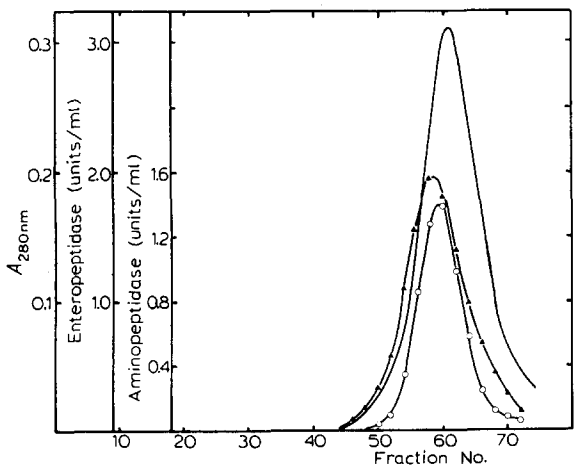

Fig. 1. Elution profile of porcine enteropeptidase on Sephadex G-200. Enteropeptidase prepared from acetone powders of duodenal fluid was filtered on a 5.0 cm × 80-cm Sephadex G-200 column equilibrated with 0.02 M Tris (acetate)/0.5 M NaCl/0.1 mM $CaCl_2$, pH 6.0. Flow rate was 50 ml per h. ——, $A^{1\,cm}_{280nm}$; ▲——▲, enteropeptidase activity; o——o, aminopeptidase activity.

7.2, containing 0.2 M NaCl and was applied to a 1.5 cm × 10.0-cm Con A-Sepharose column equilibrated with the same buffer at a flow rate of 5—10 ml per h. Enteropeptidase was not bound and appeared immediately after the void volume whereas the aminopeptidase was bound to the Con A-Sepharose and could be eluted with 150 ml 0.01 M Tris (Cl^-), pH 7.2, containing 1.0 M NaCl and 0.05 M α-methyl glucoside (Sigma Chemical Co.) (Fig. 2). Some aminopeptidase activity could be slowly eluted with 1.0 M NaCl in the absence of α-methyl glucoside so NaCl in the equilibrating buffer should not exceed 0.2 M. Recovery of the enteropeptidase activity was essentially complete. The Con A-Sepharose column could be re-used after regeneration by 2 l of 0.01 M Tris (Cl^-)/1.0 M NaCl, pH 7.2, followed by equilibration with 0.01 M Tris (Cl^-)/0.2 M NaCl, pH 7.2. Mucosal enteropeptidase showed the same behaviour on Con A-Sepharose as the enzyme prepared from duodenal fluid.

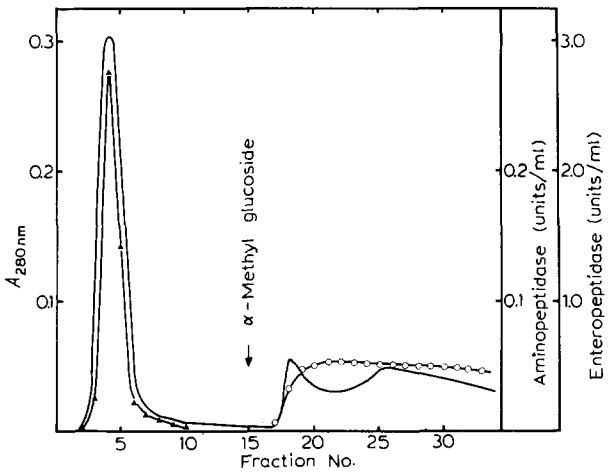

Fig. 2. Elution profile of porcine enteropeptidase on Con A-Sepharose. Enteropeptidase was applied to a 1.5 cm × 10.0-cm Con A-Sepharose column equilibrated with 0.01 M Tris (Cl^-), pH 7.2, containing 0.2 M NaCl. ▲——▲, enteropeptidase was eluted with the equilibrating buffer. ○——○, aminopeptidase was eluted with 0.01 M Tris (Cl^-), pH 7.2, containing 1.0 M NaCl and 0.05 M α-methyl glucoside. ——, $A^{1\,cm}_{280\,nm}$.

This separation of the two intestinal proteolytic activities had proved not possible by conventional ion-exchange and gel-filtration techniques without suffering large losses of enteropeptidase activity due largely to the poor resolution of the two proteins. This is in contrast to the good recovery of enteropeptidase activity with complete resolution using Con A-Sepharose. Fractionation on Con-A-Sepharose is also preferred to the use of immunoadsorption against anti-(porcine kidney aminopeptidase M) [3] because it removes not only the aminopeptidase activity but also over 80% of the contaminating protein carrying through the initial ion exchange and gel filtration fractionations described above and avoids the tedious preparation of immunoadsorbants. This procedure is now used routinely in the purification of porcine enteropeptidase both from the duodenal fluid and the mucosa and is carried out following the gel-filtration procedure.

Subsequent fractionation of the enteropeptidase preparations can be

achieved on DEAE-Cellulose (Whatman DE-32), pH 6.0, Ultragel AcA34 (LKB Produkter AB), pH 6.0, or affinity chromatography. However results obtained with affinity chromatography including *p*-aminobenzamidine-Sepharose [3] have been disappointing because of the non-reproducibility of the binding of the enzyme to the affinity support.

Because of its selectivity and simplicity Con A-Sepharose chromatography is undoubtedly the preferred method for removing the aminopeptidase activity which persistently contaminates all enteropeptidase preparations.

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