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The Purification of Human Enterokinase by Affinity Chromatography and Immunoadsorption

SOME OBSERVATIONS ON ITS MOLECULAR CHARACTERISTICS AND COMPARISONS WITH THE PIG ENZYME

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A method is described for the purification of human enterokinase from accumulated duodenal fluid by affinity chromatography using *p*-aminobenzamidine as the ligand. Resolution was greatest when glycylglycine was substituted as the spacer arm. Purification was not a one-step procedure, and some contamination, principally by the α-glucosidases, remained. Their removal was completed by immunoadsorption using antisera raised to enterokinase-free material containing these enzymes, prepared as a by-product of the purification procedure. The final preparation had an activity of 4260 nmol of trypsin/min per mg and was free of other enzymic activity tested. Amino acid and sugar analyses of the highly purified enzyme indicated an acidic glycoprotein containing 57% sugar (neutral sugars 47%, amino sugars 10%). The apparent mol.wts. and Stokes radii of human and pig enterokinase were 296000 and 316000, and 5.65 and 5.78 nm respectively. Two isoenzymes were identified for human enterokinase and three for the pig enzyme. Human enterokinase demonstrated a resistance to reduction of disulphide linkages and to sodium dodecyl sulphate binding, which may be related to the need for it to retain its integrity in the digestive environment of the upper small intestine. Antisera to highly purified pig and human enterokinases specifically inhibited enterokinase activity. Immuno-inhibition of intestinal aminopeptidase, maltase and glucoamylase by homologous antisera was not observed.

Enterokinase is the glycoprotein enzyme in the small intestine that triggers the activation of the zymogens in pancreatic juice by converting trypsinogen into trypsin, hydrolysing the lysine–isoleucine bond between residues 6 and 7 (Yamashina, 1956). In its absence the zymogens remain unactivated, digestion fails, and protein starvation results (Tarlow, 1970; Biev, 1972). Enterokinase has therefore a key permissive role in protein digestion and plays an essential part in the zymogen mechanism that prevents the pancreas destroying itself.

Despite the apparent importance of the enzyme, the purification and characterization of enterokinase from any source has not been widely attempted. Kunitz (1939) partly purified the pig enzyme by repeated ammonium sulphate fractionation; the method was later modified and improved by Yamashina (1956). Maroux *et al.* (1971), and subsequently Baratti *et al.* (1973*a*) purified pig enterokinase by fractionation on DEAE-cellulose and gel filtration on Sephadex G-100 and G-200. Although they did not comment on the presence of intestinal disaccharidase activity, they concluded that the final preparation was purified to homogeneity. Barns & Howe (1972) described the isolation of pig enterokinase from duodenal juice by ammonium sulphate fractionation followed by gel filtration on Sephadex G-200 and by DEAE-Sephadex ion-exchange chromatography, but they did not indicate the degree of purification achieved. A method has since been developed for the purification of pig enterokinase free of detectable contaminant or other enzymic activity, by using a combination of affinity chromatography and immunoadsorption (Grant & Hermon-Taylor, 1975*a*,*b*). *p*-Aminobenzamidine was selected as the ligand, which has a K_i for enterokinase of 2.0×10^{-5} M (Geratz, 1969). The specific activity of the final preparation was 2.3 times greater than the most active preparation previously reported (Baratti *et al.*, 1973*a*).

The human enzyme has never been isolated; the present paper describes the application of affinity chromatography to the purification of enterokinase from human duodenal fluid and compares some of the molecular characteristics of the enzyme with those of pig enterokinase.

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Materials and Methods

Excess duodenal fluid obtained after secretin/pancreozymin stimulation in patients undergoing routine clinical tests of pancreatic exocrine function was stored at −20°C and used as the source of the enzyme. *p*-Aminobenzamidine was kindly supplied by May and Baker Ltd., Dagenham, Essex, U.K. Crystallized trypsinogen was obtained from Boehringer Corp. (London), London W5 2TZ, U.K. Twice-crystallized trypsin was from Seravac Ltd. (Miles Laboratories, Stokes Poges, Slough, Bucks., U.K.); Sepharose 4B was from Pharmacia, London W5 5SS, U.K.; DEAE-cellulose was from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; CNBr was from Koch–Light Laboratories, Colnbrook, Bucks., U.K.; crystalline bovine serum albumin was from Hopkin and Williams, Chadwell Heath, Essex, U.K.; protein standards for molecular-weight determination and all other biochemicals were obtained from BDH Ltd., Poole, Dorset, U.K., Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., or Boehringer Corp. (London). The substitution of Sepharose 4B with the glycylglycine or 6-amino-*n*-hexanoic acid spacer arm and the subsequent coupling of *p*-aminobenzamidine was carried out by the method of Cuatrecasas (1970) as previously reported (Grant & Hermon-Taylor, 1975*a*). Ligand concentration measured by the method of Failla & Santi (1973) was 3.6 μmol/ml of gel and 3.0 μmol/ml of gel for the glycylglycine and 6-aminohexanoic acid-substituted gels respectively.

Preparation of antisera and immunoadsorbents

(i) *Antisera to selected contaminating antigens.* About 700ml of the material not adsorbed during the preparative affinity-chromatography procedure was dialysed against 10mM-sodium phosphate buffer, pH6.5. The dialysed material was stirred with 30g of DEAE-cellulose pre-equilibrated in the same buffer, allowed to settle, and the supernatant discarded. The DEAE-cellulose was washed with 150ml of 0.1M-sodium phosphate buffer, pH6.5, on a filter funnel, and the filtrate collected, dialysed against water and freeze-dried. This was resolubilized in 10ml of saline and was found to contain a trace of enterokinase activity which was removed by using 6-aminohexanoic acid-*p*-aminobenzamidine-substituted Sepharose which bound enterokinase almost irreversibly (Grant & Hermon-Taylor, 1975*b*). The antigenic material was dialysed against 2 litres of 50mM-Tris/HCl, pH8.5, and washed through a 7ml bed of this substituted gel. Material that was not adsorbed was designated 'antigen I'. Enterokinase-free material eluted by 0.5M-NaCl was designated 'antigen II'. α-Glucosidase activity was present in antigen I (1.1 μmol/min per ml).

Both antigens were freeze-dried and resolubilized in saline to a concentration of approx. 2mg/ml. Four New Zealand rabbits were each inoculated with 2.0mg of antigen I emulsified in an equal volume of Freund's complete adjuvant distributed over four sites. Two other rabbits were inoculated with antigen II in a similar manner. An intravenous boost of 400 μg of the appropriate antigen was given at 4 and 6 weeks, and serum was obtained 10 days later. Immunoglobulin was precipitated with 40% (w/v) ammonium sulphate, redissolved in saline and dialysed against 0.1M-$NaHCO_3$ containing 0.5M-NaCl. Substitution of Sepharose 4B was carried out as described by Gospodarowicz (1972). The adsorption capacity of immunoadsorbent I was 35 μg of antigen (containing maltase, 0.015 units) per ml of packed gel; immunoadsorbent II bound 30 μg of antigen per ml of packed gel.

The preparation of antisera to pig kidney aminopeptidase M and the intestinal α-glucosidases was described previously (Grant & Hermon-Taylor, 1975*a*).

(ii) *Antisera to highly purified human and pig enterokinases.* A portion (400 μg) of the highly purified human enterokinase was emulsified in Freund's complete adjuvant and two Californian rabbits were inoculated at four sites. Intravenous boosts of approximately 40 μg were given at 4 and 7 weeks, and serum was obtained 10 days later. Anti-(pig enterokinase) was prepared as described above by using a 350 μg portion of the enzyme. Intravenous boosts (20 μg) were given at 4 and 7 weeks.

Enzyme assays

The enterokinase assay was based on the improved method of Baratti *et al.* (1973*b*) and together with the assay for intestinal aminopeptidase was fully described previously (Grant & Hermon-Taylor, 1975*a*). The specific activity of enterokinase was expressed as nmol of trypsin produced/min per mg of enterokinase protein; the trypsin standard was 50% active by weight as determined by the method of Hixson & Nishikawa (1973). This was taken into account when calculating the specific activity of enterokinase. The specific activity of aminopeptidase was expressed as nmol of nitroaniline produced/min per mg of protein. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. To conserve material by avoiding protein determinations, specific activities of enterokinase and aminopeptidase were expressed as $nmol \cdot min^{-1} \cdot ml^{-1} \cdot E_{280}^{-1}$ and specific activity of maltase as $\mu mol \cdot min^{-1} \cdot ml^{-1} \cdot E_{280}^{-1}$ when monitoring the chromatographic procedures.

Disaccharidase and glucoamylase activities were measured by the two-stage method of Dahlqvist (1968); a unit of disaccharidase activity is that hydrolysing 1 μmol of disaccharide/min. Alkaline phospha-

tase activity was assessed by the method of Bessey *et al.* (1946), *p*-nitrophenyl phosphate being used as the substrate.

Analytical and characterization procedures

(i) *Polyacrylamide-gel electrophoresis.* Vertical slab-gel electrophoresis was performed by a modification of the method of Ritchie *et al.* (1966) with apparatus developed in our laboratory. Electrophoresis in the presence of sodium dodecyl sulphate was adapted from the methods of Weber & Osborn (1969) and Duncker & Rueckert (1969). Polyacrylamide gels (5%, w/v) were prepared by mixing 1 vol. of acrylamide solution (the acrylamide/bisacrylamide ratio was 29:1) with 3 vol. of 0.2M-sodium phosphate buffer, pH 7.2, containing 0.2% sodium dodecyl sulphate and 2 vol. of water containing ammonium persulphate (0.15%). The mixture was de-aerated and tetraethylenemethylene diamine (0.1%) was added. The gels were cast in tubes 8cm long and of internal diameter 4mm. Protein samples (concentration 1mg/ml) were prepared by incubating for at least 2h at 37°C in 0.01M-sodium phosphate buffer, pH 7.2, with 1% sodium dodecyl sulphate alone or with 1% sodium dodecyl sulphate and either 1% β-mercaptoethanol or 1% dithiothreitol. A portion (20–50μl) of these samples were added to an equal volume of 8M-urea before layering under the electrode buffer (gel buffer diluted 1:1 with water). A little Bromophenol Blue was added [3μl of a 1% (w/v) soln.] as the tracking dye. Electrophoresis was carried out for 2h at a constant current of 8mA/gel; the gels were then stained for protein and carbohydrate as described in the Legend to Plate 1. Relative mobility was calculated by the method of Weber & Osborn (1969). A plot of relative mobility against log(mol.wt.) was constructed for the following polypeptide standards: catalase (232000), myosin (220000), albumin trimer (204000), albumin dimer (136000), β-galactosidase monomer (132000), albumin monomer (68000), catalase monomer (58000), γ-globulin H-chain (50000), ovalbumin (43000). Values for molecular weights of standards were taken from Weber & Osborn (1969), Duncker & Rueckert (1969) and Darnell & Klotz (1972).

(ii) *Gel filtration.* A column (76.4cm×1.5cm) of Sepharose 4B (bed volume 135ml) was packed and equilibrated in 0.01M-sodium phosphate buffer, pH 7.0, containing 0.1M-NaCl. The flow rate was 5.6ml/h and fractions were collected every ½h. The void volume of the column (V_0) was determined by passage of 2ml of Blue Dextran (2mg/ml), which was detected by measuring E_{625}. The V_0 remained constant throughout the study. Eight proteins with mol. wts. in the range 60000–300000 were used as markers. The peaks for ovalbumin (43000) and bovine serum albumin (66000) were detected by measuring E_{280}. Lactate dehydrogenase (140000) was detected by following the oxidation of NADH in the presence of excess pyruvate; alcohol dehydrogenase (150000) by the reduction of NAD^+ in 2% (v/v) ethanol; catalase (232000) by following the disappearance of H_2O_2 at 240nm; pyruvate kinase (237000) by the oxidation of NADH in the presence of a lactate dehydrogenase; xanthine oxidase (275000) by following the oxidation of xanthine at 295nm; aminopeptidase M (280000) by the liberation of *p*-nitroaniline from L-leucyl-*p*-nitroanilide at 405nm. The Stokes radii of ovalbumin (2.8nm), albumin (3.65nm) and alcohol dehydrogenase (4.35nm) were available from the data of Laurent & Killander (1964). The value for xanthine oxidase (5.45nm) was calculated from the diffusion coefficient ($3.95\times10^{-7}cm^2\cdot s^{-1}$; Andrews *et al.*, 1964). The V_e corresponding to maximal activity or concentration was estimated to the nearest 1ml by the method of Andrews (1965) by which both sides of the absorption or activity peak are extrapolated to an apex. Twelve runs were made in all, by using two or three of the reference proteins each time and plots were constructed of log(mol.wt.) against elution volume (V_e), and $K_{av.}$ against Stokes radius. $K_{av.}$ was the partition coefficient between the liquid phase and gel phase and expressed as $(V_e-V_0)/(V_t-V_0)$ where V_t was the total volume of the gel bed.

Samples (2.5μg) of highly purified human enterokinase were included in three runs and 600μg of the partly purified material in two others (sp. activity 14.5nmol of trypsin/min per mg). Three samples of commercial pig enterokinase, containing approx. 2μg of enzyme were also fractionated; one sample (3.8μg) of the highly purified enzyme (sp. activity 3640nmol of trypsin/min per mg) was run separately. The use of the partly purified enzymes enabled the apparent molecular weights and Stokes radii of both pig and human maltases to be estimated at the same time. Two additional molecular-weight estimates of pig maltase and one of human maltase were made by using material separated during affinity chromatography.

(iii) *Column isoelectric focusing.* Isoelectric focusing of highly purified human enterokinase and the partially purified pig enzyme was carried out in columns (LKB 8100-1) of 110ml capacity. A 0–50% stabilizing sucrose gradient containing 1% ampholine (pH 3.5–8) was established by a gradient mixer. pH 3.5–5 ampholine solution was mixed with pH 5–8 ampholine in the ratio 5:1. Samples of enzyme were either introduced into the middle of the gradient as it was being established, or added to the gradient mixer so that the material was distributed throughout the length of the sucrose gradient. Isoelectric focusing was carried out for 48–72h and a final voltage of 700V was maintained for at least 24h. Fractions (1ml) were collected and the pH measured with a Radiometer TT-1 pH-meter. Fractions were dialysed

separately against 10mM-sodium phosphate buffer, pH7.0. The final volume of each fraction was adjusted to 3.5ml with buffer, and 0.1ml was assayed for enterokinase.

(iv) *Amino acid analysis.* Three 80μg samples of the final preparation of human enterokinase were freeze-dried in hydrolysis vials and 2ml of constant-boiling 6M-HCl was added to each. The vials were sealed under N_2 and hydrolysis was carried out for 24, 48 and 72h at 110°C. The acid was removed by rotary evaporation and the residues redissolved in lithium citrate buffer, pH2.2. Each sample was analysed on a Locarte single-column amino acid analyser. All amino acids were determined except tryptophan, which was totally destroyed by acid hydrolysis. Mean values for the number of residues were calculated for each amino acid with the following exceptions: threonine and serine were determined by extrapolation to zero-time hydrolysis, and lysine by the value at 72h, since it was maximal at that time. The values for valine and isoleucine were the same for each time of hydrolysis. Methionine was estimated by the value at 72h for methionine and methionine sulphone. Cysteine was estimated by the maximum value at 72h for cysteine and cysteic acid.

(v) *Carbohydrate content.* Carbohydrate analysis was performed by g.l.c. by using the Dunstan *et al.* (1974) modification of the method of Bhatti *et al.* (1970). A Pye series 104 chromatograph, model 24, was used and packed with 3% SE-30 on Diatomite C AW DCMS-treated. The gas phase was N_2. Enzyme protein (240μg) was mixed with 50nmol of the internal standard, mannitol, in a glass ampoule and thoroughly dried *in vacuo* in a desiccator over P_2O_5. Then 1M-methanolic HCl (0.5ml) was added and N_2 bubbled through for 30s, after which the ampoule was sealed and heated at 90°C for 24h. The acid was neutralized with silver carbonate and the material *N*-acetylated overnight with 50μl of acetic anhydride at room temperature (20°C). The supernatant was removed and the pellet twice triturated with 0.5ml of methanol; the pooled supernatants were evaporated over P_2O_5 and NaOH pellets in a desiccator. Tri-Sil (Pierce Chemical Co., Rockford, IL, U.S.A.) (30μl) was mixed with the dried material for 3min at room temperature. Two 1μl portions of this were injected into the chromatograph which was progressed from 120°C to 200°C at 1°C/min, the upper limit being held for some time after sialic acid would have emerged. Experimentally determined molar-adjustment factors were as follows: L-fucose, 1.25; D-mannose, 1.21; D-galactose, 1.18; *N*-acetylglucosamine, 1.69. The area under each curve was determined by tracing the curve, cutting out each peak and weighing the trace. All final percentages of monosaccharides refer to the dry mass (dried *in vacuo* overnight at 40°C over P_2O_5) of the glycoprotein without correction for ash.

(vi) *Immunological tests.* The abilities of various antisera to neutralize the enzyme activities of human and pig enterokinase, maltase, glucoamylase and aminopeptidase were tested. For the human, portions (50μl) of 1:10 dilutions of the antisera to antigens I and II, and highly purified enterokinase were each incubated at 37°C for 1h with samples (0.1ml) of a DEAE-cellulose extract of duodenal fluid previously dialysed against 10mM-phosphate-buffered saline, pH7.0. Each 0.1ml sample contained enterokinase (0.85nmol/min), aminopeptidase (10nmol/min), maltase (0.05μmol/min) and glucoamylase (0.01μmol/min). The incubation mixture was made up to 1ml with buffered saline, and 0.1ml was assayed for enterokinase, aminopeptidase and α-glucosidase activity. Base serum controls were also run; interference from serum trypsin inhibitor was found to be negligible at the test dilution.

For the pig, 15μl portions of antisera to pig kidney aminopeptidase M, the pig intestinal α-glucosidases and highly purified pig enterokinase were similarly incubated with 50μl samples of partially purified pig enterokinase in buffered saline. Each 50μl sample contained enterokinase (2.9nmol/min), aminopeptidase (6500nmol/min), maltase (0.4μmol/min) and glucoamylase (0.3μmol/min). Suitable dilutions were assayed for the enzyme activities.

For immunoelectrophoresis 8ml portions of 1% agarose in 0.03M-barbituric acid/sodium barbitone buffer, pH8.4, were poured on to clean glass plates (8cm×8cm×0.15cm). Samples (4μl) of highly purified human and pig enterokinase (3–30μg/ml and 2.5–250μg/ml respectively) were electrophoresed at 6V/cm for 90min at 4°C. Samples of commercial pig enterokinase, 3mg/ml (Miles Seravac Laboratories; sp. activity 4.0nmol/min per mg) and the partly purified human enzyme, 1.5mg/ml (sp. activity 14.5nmol/min per mg) were also run. The appropriate anti-enterokinase antiserum was placed in troughs cut parallel to the electrophoresed samples. Immunodiffusion was for 48h at room temperature. Similar amounts of each preparation were also run against their antisera in countercurrent immunoelectrophoresis; sample wells were 8mm apart and electrophoresis was for 90min at 6V/cm. Antigen migrated towards the cathode and immunoglobulin G to the anode.

Purification procedure

The purification procedure for human enterokinase consisted of three main stages; extraction from native duodenal fluid by adsorption on DEAE-cellulose, affinity chromatography followed by ion-exchange chromatography on DEAE-cellulose, and immunoadsorption of residual contamination.

All procedures were performed at 4°C. Duodenal fluid accumulated over a 9-month period (6 litres) was adjusted to pH6 with glacial acetic acid. DEAE-

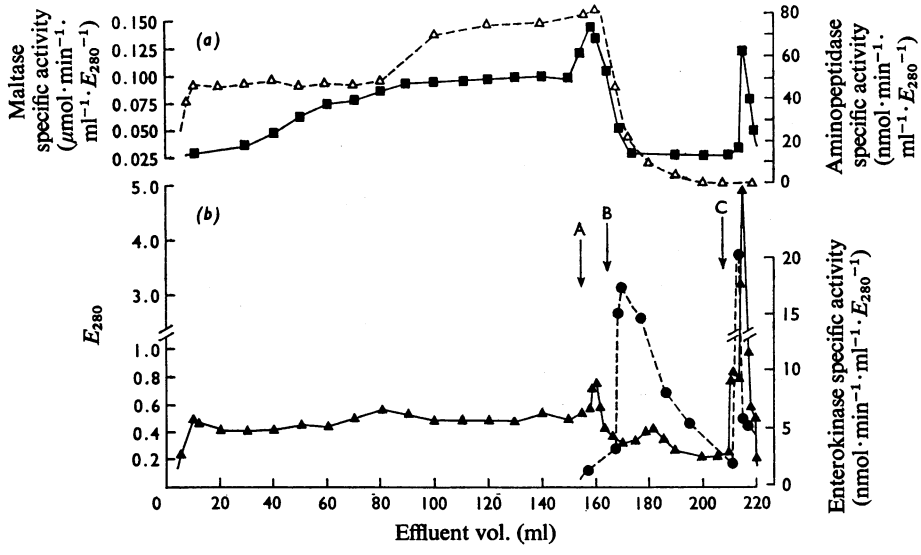


Fig. 1. *Chromatography of human enterokinase on glycylglycine-p-aminobenzamidine-substituted Sepharose 4B*

Fractions (9.8ml) were collected every 20min. The specific activity of enterokinase is expressed as nmol of trypsin·min^{-1}·ml^{-1}·E_{280}^{-1}, of aminopeptidase as nmol of nitroaniline·min^{-1}·ml^{-1}·E_{280}^{-1}, and of maltase as μmol of disaccharide·min^{-1}·ml^{-1}·E_{280}^{-1}. ▲, E_{280}; ●, enterokinase specific activity; △, aminopeptidase specific activity; ■, maltase specific activity. The arrows indicate the use of the following eluents: A, 0.05M-Tris/HCl, pH8.4; B, 0.1M-Tris/HCl, pH8.4; C, 15ml of 50% (v/v) ethylene glycol in 0.5M-Tris/acetate, pH5.6, containing 1M-NaCl.

cellulose (250g), pre-equilibrated in 10mM-Tris/acetate, pH6, was added and stirred for 1h. The pool was centrifuged at 1000*g* for 20min and the pale-yellow supernatant retained. The supernatant was dialysed extensively against a total of 100 litres of Sørenson's 10mM-phosphate buffer, pH7.6, during which a precipitate formed; this was removed by filtration through Whatman no. 1 filter paper. A further 450g of DEAE-cellulose pre-equilibrated in the same buffer was then added to the clear solution and stirred until the supernatant became negative for enterokinase. The slurry was allowed to settle, and the supernatant decanted and discarded. Enterokinase was eluted from the cellulose by washing three times with equal volumes of the same buffer containing 0.2M-NaCl. The eluate contained no tryptic activity. The combined wash (3.9litres) was dialysed against a total of 40 litres of 50mM-Tris/HCl, pH8.4.

A 60ml bed of glycylglycine-*p*-aminobenzamidine-substituted Sepharose-4B was equilibrated for 16h with 50mM-Tris/HCl, pH8.4. The enzyme solution was then pumped through the bed until enterokinase activity was detected in the eluate, which occurred after 1400ml had been applied to the column. The adsorption phase was stopped, and the column washed with 50mM-Tris/HCl, pH8.4, for 4h and 0.1M-Tris/HCl, pH8.4, for 16h. Enterokinase was then eluted, and selected fractions were assayed for enterokinase, aminopeptidase and maltase (Fig. 1). It was found that the adsorbent could not be re-used satisfactorily; fresh beds of the substituted gel were prepared, and the procedure was repeated twice with the remainder of the dialysed material. Enterokinase-containing fractions were pooled, dialysed against 10mM-sodium phosphate buffer, pH6.5, and chromatographed twice on DEAE-cellulose (Figs. 2*a* and 2*b*). The three principal enterokinase-containing fractions (Fig. 2*b*) were combined and dialysed against 2 litres of 50mM-Tris/HCl, pH8.5, containing 0.2M-NaCl.

Beds (60ml) of each immunoadsorbent (anti-I and anti-II) were equilibrated overnight with the dialysis buffer. The enterokinase solution was washed through the beds at a flow rate of 22ml/h. The eluate was retained and a portion dialysed against water and tested for the α-glucosidases. 6M-Guanidinium chloride did not regenerate the adsorbent satisfactorily, and it was necessary to pass the enzyme preparation twice more through fresh 25ml beds of immunoadsorbent I and II to remove the final traces of the α-glucosidases. Enterokinase was concentrated on a short DEAE-cellulose column in 10mM-sodium phosphate buffer, pH6.5. Sharp elution was achieved

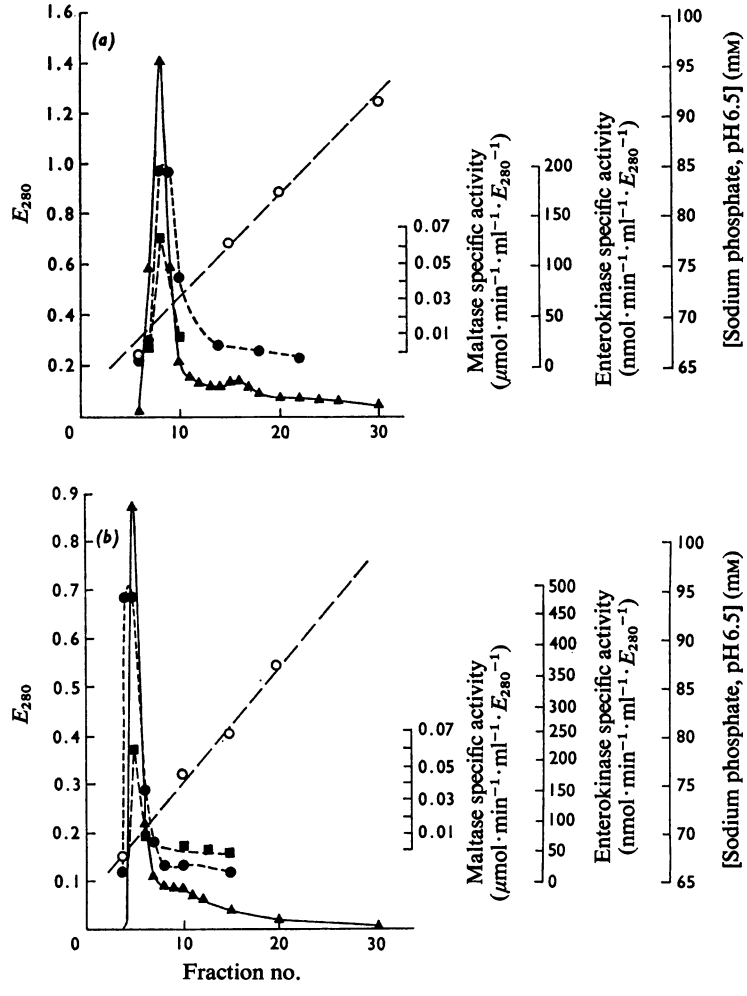


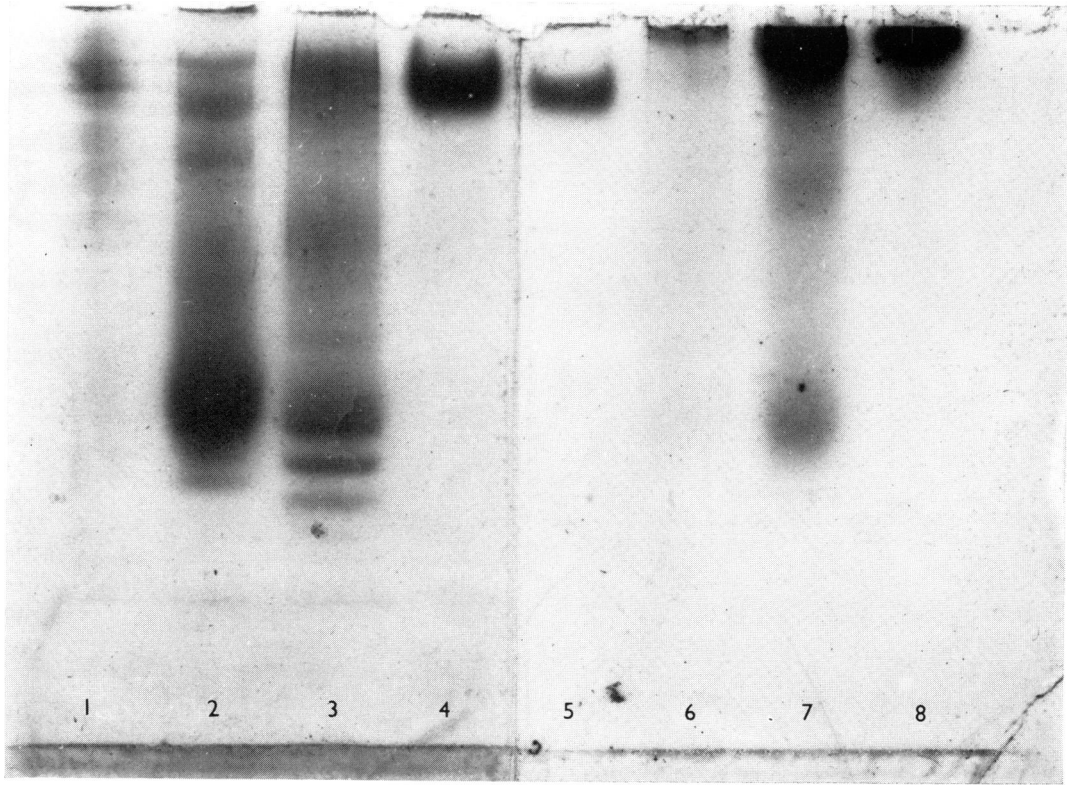
Fig. 2. *Ion-exchange chromatography of human enterokinase on DEAE-cellulose*

Enterokinase specific activity is expressed as nmol of trypsin·min^{-1}·ml^{-1}·E_{280}^{-1}. (*a*) A column of bed dimensions 34.5cm ×1.5cm diam. was equilibrated with 10mM-sodium phosphate buffer, pH6.5. Elution was by a linear gradient of 65mM–130mM-sodium phosphate buffer, pH6.5, from two 225ml reservoirs. Fractions (7.5ml) were collected every 20min. (*b*) A column of bed dimensions 10cm×2.5cm diam. was equilibrated with 10mM-sodium phosphate buffer, pH6.5. Enterokinase-containing fractions from (*a*) applied after dialysis against the equilibrating buffer. Elution was by a linear gradient of 65mM–110mM-sodium phosphate, pH6.5, from two 225ml reservoirs. Fractions (10ml) were collected every 20min. ▲, E_{280}; ●, enterokinase specific activity; ■, maltase specific activity; ○, sodium phosphate.

by 0.15M-sodium phosphate, pH6.5. The enzyme was stored in 10mM-sodium phosphate buffer, pH7, at −20°C.

Results

Table 1 summarizes enzyme activities at each stage in the purification of human enterokinase. The initial supernatant contained 777.5mg of protein from which 1.6mg of highly purified enterokinase was prepared. The yield was 60% of the total enterokinase activity. All the intestinal aminopeptidase, 96.5% of the maltase and 92.5% of the glucoamylase, were removed by affinity chromatography and ion-exchange chromatography. Removal of residual α-glucosidase activity was completed by immunoadsorption. The preparation was free of any other enzymic activity tested, including aminopeptidase,



EXPLANATION OF PLATE 1

5% Polyacrylamide-gel electrophoresis at pH 8.9

A 10cm vertical gel slab was cast by mixing 15ml of a 20% (w/v) stock acrylamide solution (acrylamide/bisacrylamide ratio, 19:1) with 30ml of 0.74M-Tris/HCl, pH8.9, 9.4ml of water, 3.75ml of 5% (v/v) *NNN'N'*-tetramethylethylene diamine and 1.8ml of 1.2% (w/v) ammonium persulphate. The reservoir buffer was 0.19M-glycine/0.025M-Tris, pH8.4 (Ritchie *et al.*, 1966). Protein (20–50μl; approx. 1mg/ml) in 0.037M-Tris/HCl, pH8.9, and containing 10% (w/v) sucrose was layered under the electrode buffer in the sample wells. Electrophoresis at 4°C was at a constant 150V for 90min. The anode is at the bottom. The gel was divided and the left half (samples 1–4) fixed in 20% (w/v) sulphosalicylic acid and stained for protein with 0.4% Coomassie Brilliant Blue R in 12.5% (w/v) trichloroacetic acid/aq. 10% (w/v) acetic acid and destained in 10% (w/v) trichloroacetic acid. The right half (samples 5–8) was stained for carbohydrate by the method of Kapitany & Zebrowski (1973). Samples (1) and (8), partly purified human enterokinase after affinity chromatography (30μg; sp. activity 121.5nmol of trypsin/min per mg); (2) and (7), antigen I (40μg); (3) and (6), antigen II (40μg); (4) and (5), human enterokinase (25μg; sp. activity 4260nmol of trypsin/min per mg).

Table 1. *A summary of the purification of human enterokinase from 6 litres of native duodenal fluid and the elimination of closely related enzymes*

The purification factor is relative to enterokinase specific activity in the DEAE-cellulose extract of native duodenal fluid.

Preparative stage	Enterokinase (sp. activity, nmol/min per mg)	Purification (fold)	Total enterokinase activity (nmol/min)	$10^{-3}\times$Total aminopeptidase activity (nmol/min)	Total maltase activity (μmol/min)	Total glucoamylase activity (μmol/min)
DEAE-cellulose extract of duodenal fluid	14.5		11240	129	384.5	34.9
Affinity chromatography and first ion-exchange chromatography	121.5	8.5	5400	0	13.9	2.5
Second ion-exchange chromatography and immunoadsorption	4260	294	6720	0	0	0

maltase, sucrase, isomaltase, lactase, glucoamylase, cellobiase, trehalase and alkaline phosphatase. The preparation was subjected to polyacrylamide-gel electrophoresis and stained for protein and carbohydrate (Plate 1).

The mean mol.wt. of human enterokinase as estimated by gel filtration (five expts.) was 296000±15000 (S.D.) compared with a mean 316000±18000 for the pig enzyme. The mean mol.wts. of human and pig maltases were 288000 ±18000 and 284000±24000 respectively. The Stokes radii of human and pig enterokinase were estimated to be 5.65±0.08nm (S.D.) and 5.78±0.11nm respectively. The values for human and pig maltase were 5.64±0.4nm and 5.53±0.12nm respectively.

The relative mobilities of the highly purified human enzyme on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis varied considerably both before and after reduction of disulphide groups. In the presence of sodium dodecyl sulphate alone the mol.wt. appeared in the range 235000–255000 in three estimations, but was 205000 and 215000 in two others. After reduction with β-mercaptoethanol, a slow-moving band in the range 235000–255000 was still observed in four runs, with a second band corresponding to an approx. mol.wt. of 150000. An additional, less-distinct, band of approx. mol.wt. 210000 was seen in those gels stained for carbohydrate. In two further runs after β-mercaptoethanol reduction, however, a slow-moving band in the mol.wt. range 200000–220000 was seen, with a second component of an approx. mol.wt. of 110000. Again an intermediate band (about 150000) was seen in the gels stained for carbohydrate. The latter pattern was also observed on the two occasions dithiothreitol was used as the reductant.

Identification of isoenzymes by isoelectric focusing

When 20μg of highly purified human enterokinase was introduced into the middle of the isoelectric-focusing column, two separate peaks of enzyme activity were identified, with pI = 4.78 and 4.90 (Fig. 3*a*). When the peak corresponding to pI = 4.90 (fractions 65–68) was pooled, dialysed successively against 10mM-sodium phosphate buffer, pH7.0, containing 0.5M-NaCl and water and re-focused, only a single peak of activity with pI = 4.89 was observed. A further 30μg of enzyme was added to the material recovered from these two experiments and distributed throughout the length of the stabilizing sucrose gradient. After isoelectric focusing for 72h, two peaks of enzyme activity were again identified with pI = 4.78 and 4.90.

Three activity peaks were consistently identified with pI values of 3.99, 4.10 and 4.21 for three samples of partially purified pig enterokinase (Fig. 3*b*). One 300mg (sp. activity 6.0nmol of trypsin/min per mg) and two 20mg (sp. activity 120nmol of trypsin/min per mg) samples were tested.

Amino acid and carbohydrate composition

The amino acid composition of human enterokinase is summarized in Table 2. The values in the Table represent the number of residues/mol of protein after correction for the sugar content and taking 296000 as an estimate of apparent mol.wt. Sugar analysis showed the enzyme to contain 57% carbohydrate (10% *N*-acetyl-β-D-glucosamine, 13% D-mannose, 13% D-galactose and 21% L-fucose; no sialic acid was detected). A slight deviation from the base line was observed at the position corresponding to *N*-acetylgalactosamine. If this was a true reading

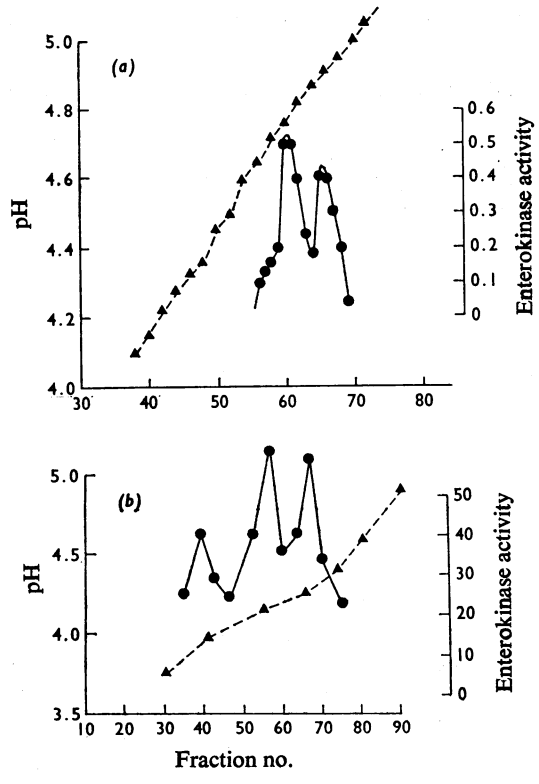


Fig. 3. *Isoelectric focusing of human and pig enterokinase*

(*a*) Isoelectric focusing of 20μg of highly purified human enterokinase. ▲, pH; ●, enterokinase activity expressed as nmol of trypsin/min per ml. (*b*) Isoelectric focusing of 20mg of partially purified pig enterokinase (sp. activity 120nmol of trypsin/min per mg). ▲, pH; ●, enterokinase activity expressed as nmol of trypsin/min per ml.

and was not due to background noise, it represented less than 1% of the number of *N*-acetylglucosamine residues/mol of enzyme, and would be equivalent to a value of less than two residues.

Serological tests

The enzyme activities of both pig and human enterokinase were completely abolished by the homologous anti-enterokinase antiserum; other antisera had no effect. By contrast, the enzyme activities of pig and human intestinal aminopeptidase, maltase and glucoamylase were unaltered by any of the antisera.

Precipitin lines were never observed when the highly purified enterokinase of each species was run against its own antiserum on immunoelectrophoresis or counter-current immunoelectrophoresis. However, anti-(human enterokinase) showed at least three components against crude human duodenal extract; pig anti-enterokinase formed two lines against the commercial preparation.

Table 2. *Amino acid composition of human enterokinase*

The number of residues/mol of protein was calculated by using 296000 as the apparent mol.wt. and corrected for 57% carbohydrate. N.D., not determined.

Residue	Number of residues/mol
Aspartic acid and asparagine	165
Threonine	89
Serine	99
Glutamic acid and glutamine	129
Proline	93
Glycine	117
Alanine	71
Valine	74
Isoleucine	62
Leucine	100
Tyrosine	32
Phenylalanine	58
Histidine	23
Lysine	58
Arginine	35
Methionine	8
Cysteine	24
Tryptophan	N.D.

Discussion

It was found that the principal enzymes associated with human enterokinase, intestinal aminopeptidase and the α-glucosidases, could not be separated by a combination of ion-exchange chromatography, gel filtration and preparative isoelectric focusing. Geratz (1969) described several amidines that inhibit enterokinase activity, and *p*-aminobenzamidine proved to be the most suitable immobilized ligand for affinity chromatography. *p*-Aminobenzamidine also inhibits trypsin ($K_i = 1.7 \times 10^{-5}$M) and was used for its purification by affinity chromatograhpy (Hixson & Nishikawa, 1973); in the present study trypsin was eliminated during the initial adsorption of the accumulated duodenal fluid on DEAE-cellulose.

During the development of the affinity system it was noted that both the length and nature of the spacer arm had a profound effect on the binding of enterokinase to the ligand. The enzyme bound almost irreversibly with 6-aminohexanoic acid as the spacer arm and the strength of binding was decreased by using a spacer arm two-methylene-residues shorter (Grant & Hermon-Taylor, 1975*b*); this effect was independent of ligand concentration (Cuatrecasas, 1972; Lowe *et al.*, 1973; Harvey *et al.*, 1974). It was

also found that enterokinase would not bind to immobilized spacer arms alone, nor to the ligand coupled to 1,4-bis-(2,3-epoxypropoxy)butane-substituted Sepharose 6B (Pharmacia Ltd., London W.5, U.K.). It seemed that the successful adsorption of enterokinase depended on a co-operation between ligand affinity and hydrophobic interaction with the spacer arm. Such hydrophobic interaction, however, also resulted in the adsorption of unwanted material. Substitution of the more hydrophilic glycylglycine was a compromise in this conflicting situation and, as with the pig enzyme, non-specific binding was decreased. A 40% loss of applied enterokinase still occurred, and the yield was not improved by decreasing the concentration of ethylene glycol to 35% (v/v) and the addition of 10mM-*p*-aminobenzamidine to the eluting buffer along the lines suggested by Lowe & Mosbach (1975).

Although about 88% of the protein in the duodenal extract was separated from enterokinase by affinity chromatography, the specific activity of the enzyme was apparently increased only twofold. This anomaly may have been due to inhibition by *p*-aminobenzamidine eluted with the desorbing buffer. The lability of the basic linkage between CNBr-activated agarose and the spacer arm was noted (Cuatrecasas & Parikh, 1972) and the adsorption capacity of the substituted gel deteriorated after storage for 1 month at 4°C. It is calculated from the data of Jennissen & Heilmeyer (1975) that less than 0.01% of the coupled ligand would need to be displaced to approach the molar concentration corresponding to the K_I in the desorbing buffer. Subsequent ion-exchange chromatography returned the specific enterokinase activity to its anticipated value.

It is likely that both ionic and hydrophobic interactions (O'Carra, 1974) contributed to the retention of the α-glucosidases, since CNBr activation of the agarose matrix may introduce a positive charge at the isourea linkage (Jost *et al.*, 1974). *p*-Aminobenzamidine had no effect on the enzyme activities of the α-glucosidases in free solution, and maltose in molar concentration did not displace maltase. Tris, however is a competitive inhibitor (Dahlqvist, 1961; Kolinska & Semenza, 1967) and 0.1M-Tris/HCl, pH 8.4, successfully resolved much of the maltase and glucoamylase from enterokinase. Desorption was time-dependent, since overnight washing was more successful than pulse elution. About 1% of the total α-glucosidase activity remained, and attempts to eliminate this by increasing the Tris concentration resulted in a progressive leaching of enterokinase from the adsorbent. The decrease in yield was unacceptable, and indicated that the limit of useful resolution of the ligand system had been reached.

It became clear that, as with some other examples of affinity chromatography (Schmer, 1972; Hixson & Nishikawa, 1973; Holroyde & Trayer, 1974), the purification of human enterokinase was not going to be achieved in a one-step procedure. The direction of the investigation therefore changed to the isolation of related contaminants free of enterokinase. In this we were able to exploit the ability of the 6-aminohexanoic acid-*p*-aminobenzamidine-substituted gel to bind enterokinase almost irreversibly. The need to present a small number of antigens/immunizing dose, to minimize suppression of antibody formation by non-specific antigen (Pross & Eidinger, 1974), was fortuitously served, since the unabsorbed material and the enterokinase-free fraction eluted by 0.5M-NaCl each contained about six different components. The strength of the resulting antisera was, however, not great, and might have been improved by subdividing the antigens still further.

The combination with immunoabsorption means that the specificity of a ligand system in affinity chromatography need not be absolute for the purification to homogeneity of a desired macromolecule provided: (*a*) The affinity of the ligand complex is greatest for the desired species and the number of contaminants is minimized by optimal resolution; and (*b*) adsorbing antisera contain immunoglobulin specific for each residual contaminating antigen.

Each technique compensates for the limitations of the other, affinity chromatography decreasing the number of antigens to be presented, and immunoadsorption overcoming the lack of absolute specificity. The mode of action of each is the reciprocal of the other, affinity chromatography retaining the desired species and immunoadsorption the unwanted material. This is preferable to attempting to displace desired antigen in its biologically active form from immobilized specific antibody, the difficulties of which have not yet been completely resolved (Gospodarowicz, 1972; Murphy, 1974).

The highly purified enzyme, extracted from duodenal fluid accumulated from about 40 patients, appeared as a single, well-defined band on polyacrylamide gel after 90min or 6h electrophoretic separation. This apparent homogeneity contrasted with the dispersed appearance of the pig enzyme (Grant & Hermon-Taylor, 1975*a*), which was probably due to the displacement of terminal sugars in the relatively harsh conditions used to prepare the commercial extract. The mild conditions employed for the extraction of the human enzyme were reflected in a final activity that was 17.5% greater than that of pig enzyme. The human enzyme contained no other enzymic activity tested, and it was concluded that its homogeneity could best be assessed by the demonstrable lack of heterogeneity, as discussed by Gibbons (1972).

The high sugar content was probably the reason for the variable behaviour of human enterokinase on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, since the carbohydrate-free marker proteins migrated consistently. Although Dunstan *et al.*

(1974) indicated that mol.-wt. estimation of glycoproteins by electrophoresis in the presence of sodium dodecyl sulphate by the method of Weber & Osborn (1969) may give consistent results, the value for human enterokinase ranged from 210000 to 245000. This agrees with the estimate of 220000±30000 obtained by Maestracci *et al.* (1975) under similar conditions; the variability is presumably due to incomplete sodium dodecyl sulphate binding to the polypeptide core owing to interference from the surrounding oligosaccharide chains. Similar variations occurred after reduction of human enterokinase with β-mercaptoethanol or dithiothreitol. The slowest migrating band corresponded to that obtained in the presence of sodium dodecyl sulphate alone, suggesting that reduction was incomplete. Such resistance to reduction and the binding of sodium dodecyl sulphate might be related to the resilience the enterokinase molecule has been required to evolve to survive in the fiercely digestive environment of the upper small intestine.

Estimations of the mol.wts. of pig and human enterokinase by gel filtration are unreliable, since the relationship of log(mol.wt.) to elution volume (V_e) can be satisfactorily applied only to globular proteins (Andrews, 1965). However, the Stokes radius of enterokinase could be accurately compared with the four protein standards where this value was available, since the amount of chromatographic bed accessible to each was dependent on their molecular volumes in aqueous solution and given by the K_{av}. (Laurent & Killander, 1964). The apparent mol.wt. of the pig enzyme differed by 100000 from the value of 212000 previously obtained by fractionation through Sephadex G-200 (Baratti *et al.*, 1973*a*). The intestinal α-glucosidases of each species were very similar in apparent mol.wt. to enterokinase, confirming the unsuitability of gel filtration as a method for their separation.

Illingworth (1972) described the apparent identification of multiple forms of yeast isocitrate dehydrogenase by isoelectric focusing. He concluded that they were artifacts due to the binding of acidic components of the ampholine solution to the enzyme during the passage of current through the column, and were related to the protein/ampholine ratio. This is unlikely to be the explanation for the three isoenzymes consistently identified for pig enterokinase, since the pI values were constant at widely different protein/ampholine ratios. The two isoenzymes clearly identified in the highly purified human preparation under different experimental conditions were also independent of this ratio. The re-focusing of one isoenzyme did not alter its isoelectric point, nor did it split into the two forms which might have been expected if the ampholytes were binding non-specifically. The different pI values for each isoenzyme may be due to variations in the polypeptide core or carbohydrate fraction or both. Saini & Done (1972) demonstrated three isoenzymes for alkaline phosphatase in the rat intestinal brush-border and suggested that the source tissue contained cells in different stages of maturation. If the biosynthesis of enterokinase follows a similar pattern, it could explain the presence of three pig isoenzymes, since the commercial material would have contained components representative of several cellular stages. The human enzyme however, was prepared from duodenal juice, and the enterokinase released into the lumen was likely to be the final product of such a pathway. The generation of artificial enzymes by chemical mistreatment during the preparative steps should also be considered. It is likely that the pig enzyme, rather than the human one, would be made microheterogeneous in this way.

Analysis of the human enzyme indicated a composition of 57% carbohydrate and 43% amino acid residues. Kelly & Alpers (1973) reported that the human intestinal brush-border enzymes, maltase/glucoamylase (maltase II) and sucrase, contain some 22–31% and 47–52% of sugar respectively. Fucose, mannose, galactose and *N*-acetylglucosamine are common to these and to enterokinase, but sialic acid is absent. *N*-acetylgalactosamine, which is apparently well represented in the disaccharidases, is negligible in human enterokinase. Dische (1963) suggested that there is a reciprocal relationship between the amounts of fucose and sialic acid as terminal sugars of the oligosaccharide chains, and that the uncharged fucosyl residues are more hydrophobic in character than sialic acid. The high fucose content of both enterokinase (21%) and maltase/glucoamylase (10–15%) may have accounted for their non-specific hydrophobic binding to the various adsorbents tested. Mannose, *N*-acetylglucosamine and galactose are typical constituents of glycoprotein enzymes and the glycopeptide linkage in human enterokinase is likely to be an *N*-glycosyl bond between *N*-acetylglucosamine and the amido group of asparagine (Spiro, 1970). The peptide sequence Asn-X-(Thr/Ser), with X as any amino acid, appears to be essential for the coupling of the hexosamine to asparagine (Marshall, 1974). Serine and threonine constitute about 15% of the total amino acids of enterokinase, indicating that there are sufficient residues to satisfy this requirement. Glutamate and asparate, together with their amides, comprise about 25% of the total residues, and this would account for the acidic isoelectric points determined for the isoenzymes. The relatively large amount of proline (7.5%) would suggest that a significant proportion of the polypeptide chain is not in the helical form; the low cysteine content (<2%) would indicate that few disulphide bonds are found in the tertiary structure both intra- and intermolecularly.

There was insufficient highly purified pig entero-

kinase to carry out an analysis of its composition. Baratti *et al.* (1973*a*) reported that the sugar content is 37%, consisting of 2% sialic acid, 20% neutral sugars and 15% amino sugars. The ratios of the individual amino acid residues were comparable with the observations for the human enzyme, which may indicate some similarity in the polypeptide cores of the two species.

Although specific antibody to both pig and human enterokinase were demonstrably prepared, precipitin lines were not obtained when each was tested against its own sensitizing antigen in immuno- and counter-current electrophoresis. Each antiserum, however, formed precipitin lines against the appropriate crude extract, despite the fact that both enzymes were prepared to homogeneity within the limits of the tests used to assess purity. An explanation compatible with these observations would be that antibodies to the enzymes from each species are non-precipitating (Arnon, 1967) but that incomplete cross-reaction was occurring with other intestinal components of the same species. Examples of cross-reaction are well documented in the literature (Landsteiner, 1946; Dodd, 1952; Fuks *et al.*, 1974). The immuno-inhibition of pig and human enterokinase is likely to be due to steric hindrance, with antibody masking the active site preventing the access of large-molecular-weight substrates (Arnon, 1967). This cannot be so for intestinal maltase and aminopeptidase, since antisera to these enzymes did not abolish their enzymic activities but bound them when immobilized on agarose.

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