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Identification of pancreatic enzymes in human duodenal contents

In animals, pancreatic enzymes and zymogens have been studied in great detail by many groups of workers[1–8]. Techniques such as separation on charged columns and polyacrylamide gel electrophoresis have been applied for the study of bovine[1–3], porcine[4–6], canine[4], and rat[7,8] pancreatic enzymes.

In man, pure pancreatic secretions are available only in such rare situations when an external pancreatic fistula is formed or from patients undergoing pancreatic surgery. Therefore, only a few detailed studies concerning human pancreatic enzymes have been made so far[9].

Since human pancreatic juice is not easily accessible, it is of interest to study the occurrence of human pancreatic enzymes in a secretion which is more readily available, duodenal contents.

In the present study, pancreatic enzymes occurring in human duodenal juice were separated by a rapid and simple technique (micro-electrophoresis on cellulose polyacetate membranes) and identified using histochemical stains and elution procedures. This was done after stimulation of exocrine pancreatic secretion with the gastrointestinal hormones pancreozymin and secretin.

Under these conditions normal human duodenal juice contains at least eleven major protein components, eight of which were identified as pancreatic enzymes. It is suggested that these methods are used for the investigation of pancreatic disorders in man and in particular for the study of single enzyme deficiencies.

Human duodenal juice was collected from fasting healthy adults using a three-lumened duodenal tube in order to avoid contamination of duodenal fluid with gastric contents[10]. Samples for biochemical analysis were collected during 20 min after the intravenous injection of 2 units/kg body wt. pancreozymin (Boots) and for 30 min after intravenous injection of 2 units/kg body wt. secretin (Boots). The specimens were immediately treated with DFP (final concn., 10^{-3} M) and stored for not more than 2 days at −15° before analysis. In those instances where enzymes which are inhibited by DFP (trypsin, chymotrypsin), had to be analysed, DFP treatment was omitted and the samples were subjected to electrophoresis immediately after their collection.

The conditions of electrophoresis and the materials used are described in the legend to Fig. 1. After electrophoretic separation, the cellulose polyacetate strips were cut longitudinally and one half of the strip was stained for protein with a solution of 0.1% nigrosine in 2% acetic acid containing 10% glycerol. The site of the enzymatic activity was located on the other half of strip by covering it with two reagent strips. The first of these was soaked in the specific chromogenic substrate and the colour was developed with a second strip which had been soaked in the coupling reagent (fast blue salt B, Merck). The colour developed within a few minutes and photomicrographs were taken immediately since the colour tended to fade as the strips dried.

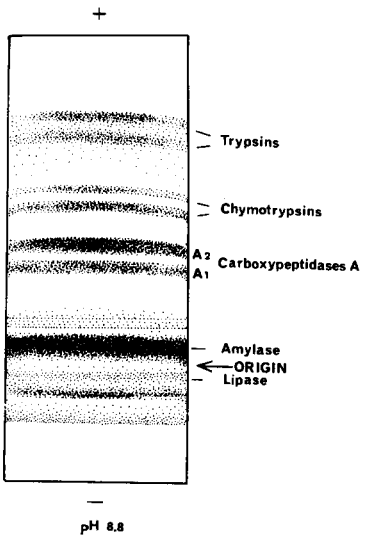
For the histochemical stains, the following specific substrates were used: *N*-Carbo-β-naphthoxy-DL-phenylalanine (Sigma) (4 mg/ml methanol diluted 1:3 with 0.2 M Tris buffer, pH 7.8) for the detection of carboxypeptidase A activity[11]; *N*-benzoyl-DL-phenylalanine-β-naphthol ester[12] (Mann) (4 mg/ml acetone, diluted 1:3 with

0.05 M Tris buffer, pH 7.0, containing 0.02 M $CaCl_2$) for the detection of chymotrypsin; and *N*-benzoyl-DL-arginine-β-naphthylamide·HCl[13] (Mann) (4 mg/ml methanol, diluted 1:2 with 0.05 M Tris buffer, pH 8.0, containing 0.02 M $CaCl_2$) for the identification of trypsin.

The amylase activity was detected using the method of TREMBLAY[14]. The starch slide was prepared using a 4% solution of starch in 0.05 M phosphate buffer (pH 6.9) and the electrophoresis strip was placed in contact with the slide for 10–20 min. After staining with Schiff–periodate stain a clear band on a red background indicated the site of enzyme activity.

For the localisation of lipase activity, the zones corresponding to a protein band were eluted from one half of the strip and lipase activity was determined by the method of MURCHIS-MOUREN, SARDA AND DESNUELLE[15]. A microtitrator (Ingold, Zürich) was used and the electrophoresis strip was introduced directly into the chamber of the titrator which contained 2 ml substrate.

Fig. 1 shows the protein components of DFP-treated normal human duodenal juice collected after combined stimulation with pancreozymin and secretin. Eleven protein components are detectable, eight of which have been identified as pancreatic enzymes. Beginning with the most anionic and ending with the cationic enzymes,



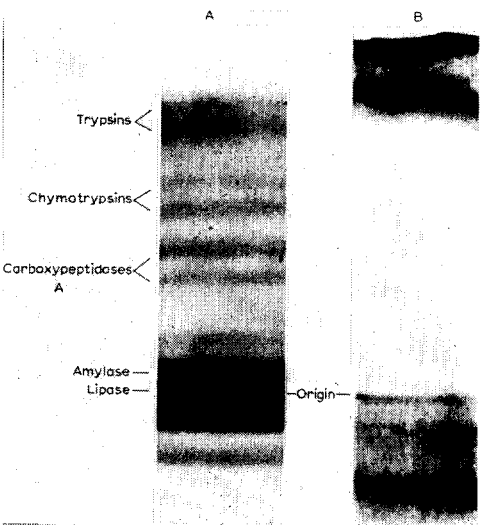


Fig. 1. Diagram drawn after a photomicrograph showing the typical protein pattern of normal human duodenal juice. The sample was collected after combined stimulation with pancreozymin and secretin of exocrine pancreatic secretion. 10 μl duodenal juice (treated previously with DFP as indicated in the text) were applied to a cellulose polyacetate membrane (Sepraphore III, Gelman, width 2.5 cm). The buffer was 0.025 Tris–barbital–sodium barbital, pH 8.8. 1.5 mA per strip were applied for 40 min at room temperature. Protein stain with nigrosine and identification of enzymes as indicated in the text.

Fig. 2. Photomicrographs showing protein patterns of (A) normal DFP-treated duodenal juice after stimulation of pancreatic secretion with pancreozymin and secretin; (B) duodenal juice from a patient with exocrine pancreatic insufficiency. The sample used for the electrophoresis from the patient showed no detectable enzymatic activity except traces of trypsin-like and chymotrypsin-like activity, measured against the specific substrates mentioned in the text.

these are: two forms of "trypsin", two forms of "chymotrypsin", two carboxypeptidases A (A_1 and A_2), one amylase and one lipase. All samples of duodenal juice collected after combined stimulation of exocrine pancreatic secretion showed essentially the same pattern in electrophoresis. There was no difference in the protein pattern between samples collected after combined stimulation compared with samples collected after stimulation with pancreozymin alone.

In Fig. 2 the electrophoretograms of normal human duodenal juice and of juice from a patient suffering from exocrine pancreatic insufficiency* are compared. In the patient, none of the characteristic bands of normal duodenal juice are seen. In stead, two heavy bands with high electrophoretic mobility appear, which are not seen in the normal juice.

It appears, from these results, that it is feasible to use human duodenal contents as the source of enzymes for the study of human pancreatic function and that electrophoretic separation of the proteins of duodenal juice with a rapid and simple microtechnique (cellulose–polyacetate membranes requiring only 10 μl sample) reveals marked differences between juices obtained from patients with pancreatic insufficiency and juices collected from normal individuals. Furthermore, several forms of endopeptidases and carboxypeptidase A are detected with this method in normal duodenal juice. One main difficulty exists however in the interpretation of "zymograms" from duodenal juice: proteolytic enzymes are fully activated (in contrast to pancreatic juice where they occur as zymogens) and rapid autodigestion of the juice is likely to take place. It is essential, therefore to inactivate trypsin and chymotrypsin by DFP treatment immediately after the collection of the sample or to proceed with the electrophoretic separation as soon as the sample has been collected. The duplicity of carboxypeptidase A has been shown to be the result of the action of trypsin an the A_1 form of the enzyme[17,18]. It remains to be elucidated whether the enzymes having trypsin- and chymotrypsin-like substrate specificity represent different activation products of a single zymogen or discrete enzymatic entities. The anionic nature of "trypsins" and "chymotrypsins" would agree with the observations of KELLER AND ALLEN[9] who studied these enzymes in pure human pancreatic juice; however, KELLER AND ALLEN found no evidence for a second form of human chymotrypsin. One form of pancreatic amylase was revealed in this study, which would agree with the observations of LEARCHY *et al.*[19]. In contrast, KELLER AND ALLEN[9] have been able to demonstrate three forms of human pancreatic amylase using an anionic polyacrylamide gel system. The cationic nature of a single lipase is in agreement with the findings of KELLER AND ALLEN in human pancreatic juice.

The obvious difference between the electrophoretic pattern of the patient with exocrine pancreatic insufficiency compared with normal duodenal juice, would indicate that this method is in fact valuable in the diagnosis of exocrine pancreatic insufficiency. ZOPPI *et al.*[20] have recently shown, that large amounts of serum proteins can be found in duodenal contents of patients suffering from exocrine pancreatic insufficiency. The two heavy bands observed in the electrophoretogram of our patient are likely therefore to be serum proteins. This phenomenon is probably due to an

* The clinical features of this patient (case No. 4 in ref. 10 and case No. 11 in ref. 16) have been described by BURKE *et al.*[16] and results concerning his pancreatic function have been published by HADORN *et al.*[10].

accumulation of serum proteins in the duodenum, in the absence of the digestive power of pancreatic proteinases.

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Peptide substrates for a proteinase of *Clostridium histolyticum*

Together with collagenases[1], a thiol-activated proteinase[2], and peptidases[3], *Clostridium histolyticum* produces in its culture medium a proteinase that is optimally active in the presence of calcium salts and for which no synthetic substrates are known[4]. The only information on its specificity was presented in a comparison of N-terminal amino acids of peptides released from gelatin by a fraction containing collagenase and this proteinase[5]. These data suggested that the proteinase specifically