Purification and characterization of a hormone-like factor which inhibits cholera secretion

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A factor which inhibits intestinal hypersecretion induced by cholera toxin was studied. The factor was extracted from intestinal mucosa or pituitary gland of pig. It has an isoelectric point of pH 4.7 ± 0.1 at 10° C and showed a weak affinity to dextran gel and a strong affinity to agarose gel. From agarose gel the factor was eluted with high concentrations of D-galactose or α-methyl-D-glucose, while D-glucose and lactose were less effective. After purification more than 2000 times by isoelectric focusing and gel chromatography, the factor was shown by SDS–polyacrylamide gel electrophoresis to be a protein consisting of subunits of molecular mass 30, 17 and possibly 15 kDa.

Enterotoxin *Cholera* *Gastrointestinal peptide* *Peptide hormone* *Intestinal secretion* *Pituitary gland*

1. INTRODUCTION

Cholera toxin induces diarrhoea by increasing the permeability of water and electrolytes in the small intestine [1]. However, man as well as animals previously exposed to the toxin show a reduced diarrhoea response to a second dose of toxin [2]. After several exposures the intestinal epithelium becomes highly resistant, and does not respond with diarrhoea to toxin challenge. This resistance seems to be a function of desensitization of the intestinal adenylate cyclase [3] in combination with the production of an anti-diarrhoeic factor [4].

The factor is a heat-labile macromolecule (M_r 10000–50000), which effectively inhibits fluid secretion by cholera toxin in rat intestine [4]. When toxin-resistant rats were investigated for the presence of anti-diarrhoeic factor in different organs, only the intestine and the central nervous system contained detectable amounts. The highest concentrations were found in the pituitary gland, and the content was proportional to the number of exposures to cholera toxin.

This investigation deals with a similar factor of pigs, naturally found in the small intestine and in the pituitary gland. Our aim was to purify the antisecretory factor, and for this purpose we used isoelectric focusing (IEF), gel filtration and affinity chromatography. After purification, the factor was analysed by polyacrylamide gel electrophoresis (PAGE) in presence of SDS.

2. MATERIALS AND METHODS

2.1. *Extraction*

Pituitary glands from freshly slaughtered piglets (6–7 months old, mixed sexes) were free-dissected, frozen in liquid nitrogen and stored at −80°C until use. From the same animals intestinal mucosa from proximal jejunum was scraped off the muscularis layer with help of a scalpel, immediately frozen in liquid nitrogen from which it was thawed and processed the same day. The tissue was supplied with 2–3 ml 0.15 M NaCl–0.05 M sodium phosphate, pH 7.2 (PBS), per g wet wt and then homogenized for 2 × 15 s at 5000 rpm in an Omnimixer (Sorwall) and a Dounce glass

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homogenisator (5 strokes, loosely fitting). After a 2-fold centrifugation for 15 min at 15000 rpm the clear supernatant was used as starting material ('crude extract') for further purification.

2.2. *Purification*

Ultrafiltration was performed with XM50 and PM10 membranes (Amicon); they had a cut-off effect for spherical proteins of molecular mass ~50 and ~10 kDa, respectively.

Preparative IEF was performed in a 440-ml column (LKB) as described [5]. A linear sucrose gradient was formed by mixing 180 ml of 40% sucrose with 180 ml of the samples. Totally 9 ml of a 40% solution of Ampholine (pH 4–6) (LKB) was supplied. As anode electrode solution 0.1 M glutamic acid–0.5 M phosphoric acid and as cathode electrode solution 0.1 M β-alanine–0.5 M ethanolamine was used. At the start 550 V, 40 mA was applied; the run was finished after 20 h at 700 V, 6 mA. The separated material was collected in 17–18-ml fractions and the pH measured at 10°C in each fraction. The fractions were dialysed against PBS, concentrated 3–5 times against polyethylene glycol and their anti-diarrhoeic activity assayed (see below).

Gel filtration on Sephadex G100 Fine (Pharmacia) was performed on pooled fractions from the IEF of extracts of pituitary glands. Three columns of diameters 1.25, 2.5 and 5 cm, respectively, were used. Their gel contents were 78, 326 and 1200 ml, respectively (approx. flow rate 18 ml/cm^2 per h). The absorbance at 280 nm was registered in each fraction while the anti-diarrhoeic activity was assayed in pooled or individual fractions (see fig.2).

Affinity chromatography on agarose gel (Sepharose 6B, Pharmacia) was performed either on crude pituitary extracts or on pituitary material purified by IEF and gel filtration. Columns with a bed volume of 1 or 3 ml, respectively, were used. In the small columns 1.5 ml of the crude pituitary extract was applied and after washing with 3 ml of PBS the active material was eluted by applying 3 ml of 0.5 M sugar dissolved in PBS (see table 1, experiment 3,4). To the larger column 20 ml of the partially purified factor was applied. The material thus attached to the gel was step-wise eluted with increasing concentrations of α-methyl-D-glucose, 6 ml per fraction as described in table 1. All sugars were of analytical grade and each fraction was dialysed against PBS before testing of the anti-diarrhoeic activity.

2.3. *Activity test*

The anti-diarrhoeic activity was tested in rats as described [6]. In brief cholera toxin-induced fluid secretion was estimated in an intestinal loop. The toxin dose used was choosen to give a half-maximal secretion (normally about 1 μg). The test sample was injected intravenously and its effect on the cholera secretion was related to that of controls given PBS intravenously.

2.4. *SDS–PAGE*

SDS–PAGE was performed as in [7] using 10% polyacrylamide. Reference proteins covering a molecular mass range of 14–90 kDa were obtained from Pharmacia.

3. RESULTS AND DISCUSSION

The crude homogenate of porcine pituitary gland caused a pronounced inhibition of intestinal fluid secretion by cholera toxin. A total inhibition was obtained using a dilution of 1:5–1:10 of the homogenate while a dilution of 1:50–1:100 caused some 50% inhibition.

The homogenate of intestinal mucosa was less effective, causing 50% inhibition at a dilution of 1:10. Due to the large amount of the intestinal mucosa, the total content of the inhibitory factor was probably equal or higher in the intestine in comparison with that of the pituitary gland. The concentration, however, was much higher in the pituitary gland, which therefore was used for purification of the factor.

Ultrafiltration on defined membranes revealed that the pituitary factor easily passed membranes with a cut-off effect at M_r 50000 but was totally retained by a membrane with cut off effect at M_r 10000.

Preliminary separations on IEF of the pituitary extract revealed that all of the inhibitory activity focused around pH 5. The subsequent separations were therefore performed in carrier ampholytes with a pH range between 4 and 6. As shown in fig.1, the inhibitory activity of the intestinal as well as the pituitary material was focused about pH 4.75 (at 10°C). The similarity in isoelectric point

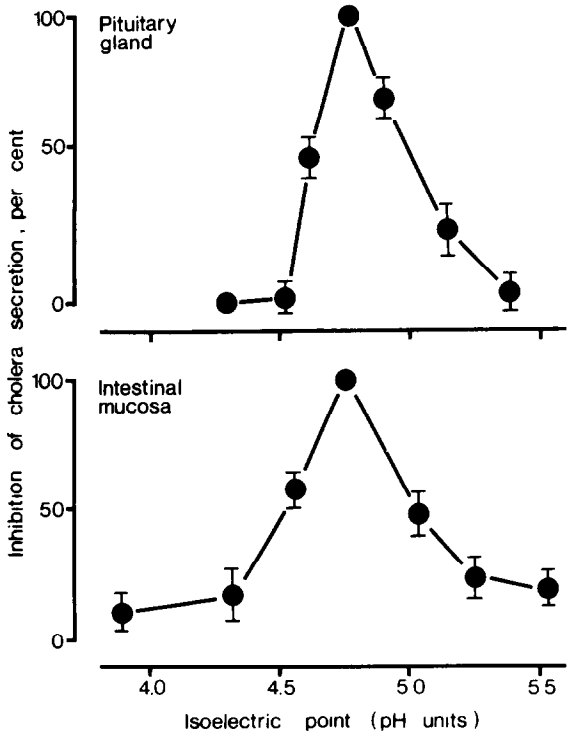

Fig.1. The anti-diarrhoeic effect of extracts from porcine pituitary gland or intestinal mucosa after separation by isoelectric focusing. The electrofocusing was performed in a preparative column containing ampholine, pH 4–6, in a sucrose gradient. After measurement of pH and dialysis, each fraction was injected intravenously in rats. Cholera toxin-induced fluid secretion was subsequently measured in the small intestine of these rats. The inhibition of the cholera secretion is expressed as the mean ± SE of 3 animal groups.

suggests that the same molecule is responsible for the inhibitory activity in the two organs.

The pituitary material was further purified by gel filtration on Sephadex G100. As shown in fig.2 the inhibitory factor eluted in two peaks: the first one at a volume corresponding to a globular protein of M_r 15000 and the second at an apparent $M_r < 10000$. When the amount of the applied material or the gel volume was varied, the proportion of the two peaks was changed. A large ratio of applied amount/gel volume favoured the first peak while a low ratio favoured the second peak (which then contained almost all the activity). The first peak was narrow while the second one was

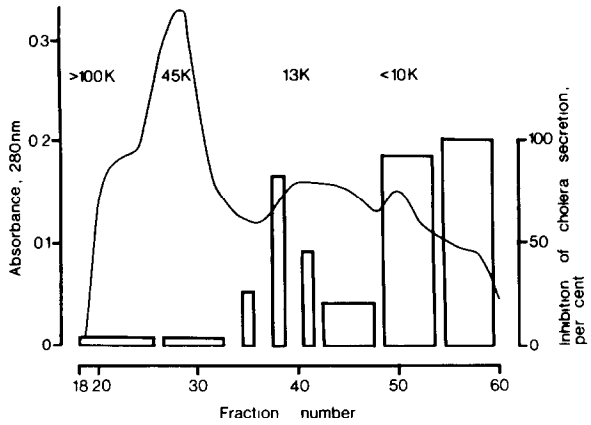

Fig.2. Separation by gel filtration on Sephadex G 100 of the factor. The absorbance at 280 nm (curve) and the inhibition of cholera secretion (bars) were measured. The latter assay was performed as described in fig.1.

broad, and extended much behind the reference for low molecular masses, diethyl barbituric acid. The filtration pattern in Sephadex G100 suggests that the anti-diarrhoeic substance interacted with dextran gel, the two peaks probably representing two different affinity states.

The third purification step was exerted on small columns with agarose gel. The anti-diarrhoeic activity was totally absorbed by the agarose gel even when crude extracts of pituitary glands were tested. As shown in table 1, various sugars and sugar-derivates were tested for their capacity to elute the anti-diarrhoeic activity from the gel. Galactose and α-methyl-D-glucoside eluted almost all the activity, glucose eluted some of the activity while lactose was ineffective.

Agarose consists mainly of the repeating disaccharide unit $\longrightarrow$3) D-galactopyranose (β1$\longrightarrow$4)3,6-anhydro-L-galactopyranose (α1$\longrightarrow$ [8]. Since lactose, D-galactopyranose-(β1$\longrightarrow$4)-D-glucose, had no effect the factor probably does not bind to the β-D-galactose residue of the gel. Alternatively, the 3,6-anhydro-L-galactose residue or some minor constituent of the gel may bind. Structures larger than monosaccharides might also be recognised by the factor. The lectin-like binding of the factor is possibly important for its transport with body fluids and uptake by the intestinal mucosa.

Highly purified anti-diarrhoeic factor was achieved by the following 3 steps: (1) IEF, (2) gel

Table 1

The affinity of the antidiarrhoeic factor to agarose gel

Sugar		% inhibition of CT-induced fluid secretion	
		experiment 1	experiment 2
Glucose,	0.5 M	34 ± 8	33 ± 5
Galactose,	0.5 M	78 ± 3	58 ± 3
Lactose,	0.5 M	0 ± 2	11 ± 11
αMe-glucoside,	0.5 M	86 ± 7	71 ± 12
		experiment 3	experiment 4
Direct eluate		0	0
αMe-glucoside,	0.1 M	7 ± 20	30 ± 10
	0.3 M	46 ± 12	61 ± 5
	0.9 M	>95	>95
	1.5 M	2 ± 27	30 ± 18

Elution with different sugars of the factor attached to columns with agarose gel. In experiment 1 and 2 different columns were used for each sugar. In experiment 3 and 4 the factor was eluted stepwise from one column by applying increasing concentration of αMe-glucoside

filtration, and (3) affinity chromatography on agarose. As shown in table 2, 100 pituitary glands were used and the crude homogenate contained $1017 \times 0.84 = 854$ ED_{50} doses (each dose was able to cause a 50% inhibition of the cholera secretion). After fractionation by IEF the protein content was less than 1/10 of that of the starting material. The specific activity had increased 15-times (from 0.84 to 12 ED_{50} doses per mg protein) with a yield of 63% of the original activity. Gel filtration on Sephadex G100 (peak 2) increased the specific activity about 1300-times in relation to that of the crude homogenate. Considering the high degree of purification during this step the yield, 39%, was very good. The affinity chromatography on agarose gel increased the specific activity to more than 2000-times that of the original material. Unfortunately more than 5/6 of the activity was lost during this step leaving only small amounts of protein for the final analyses. Hopefully, a sugar derivative with higher eluting capacity than α-methyl-glucoside will be found in order to get a higher yield during the chromatography on agarose.

The purified factor was finally analysed by SDS–PAGE. After partial purification with IEF more than 20 components, corresponding to molecular masses between 12 and 100 kDa, were obtained (fig.3). Further purification with gel filtration resulted in 8–10 bands in the gel (not shown). After the final chromatography on agarose gel only 2 or 3 bands were obtained. As shown in fig.3, the two larger bands were equally stained while the smallest one varied in amount from preparation to preparation. The estimated molecular masses for the 3 components were 30, 15 and 13 kDa, respectively. Attempts to stain the purified factor with Schiff reagent or Alcian blue failed, which suggests that no carbohydrate is present in the factor.

Thus, the anti-diarrhoeic factor from pig pituitary gland seems to be a protein of an isoelectric point of pH 4.7–4.8 and built up of subunits of molecular mass 30, 15 and possibly also 13 kDa. The protein has lectin-like characteristics and interacts with agarose as well as with dextran gels.

Table 2

Purification of the anti-diarrhoeic factor extracted from pituitary gland of pig

	Protein content (mg)	Inhibitory activity (ED_{50} doses/mg)	Yield of activity (%)
Crude homogenate	1017	0.84	
Isoelectric focusing	88.7	12	63
Gel filtration	0.495	1100	39
Affinity on agarose	0.027	1800	6

The crude homogenate from 100 pituitary glands was purified in 3 subsequent steps. Each preparation was tested as described in fig.1. The inhibitory activity is expressed as the number of doses per mg protein giving 50% inhibition (ED_{50})

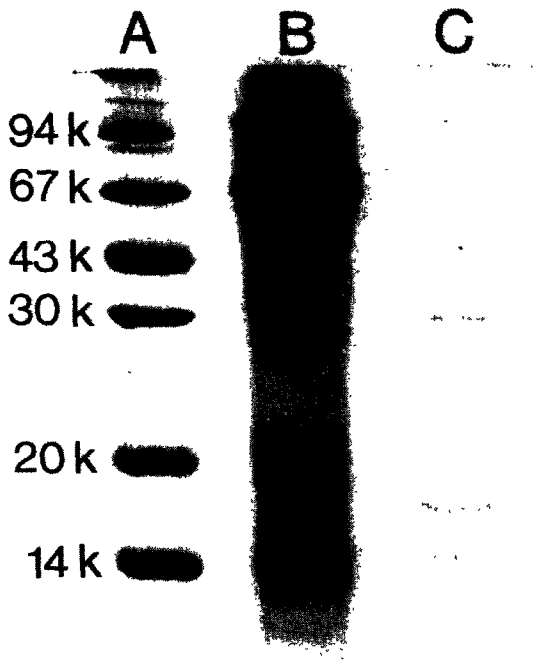

Fig.3. Analysis by SDS–PAGE of the factor. (A) Reference proteins with molecular masses between 14 and 90 kDa, (B) fraction pH 4.6–4.9 in isoelectric focusing, (C) the factor purified by isoelectric focusing, gel filtration and affinity chromatography.

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