Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum

Isolation of the peptide

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A 37 amino acid-peptide has been isolated from porcine jejuno-ileum on the basis of its glucagon-like activity in liver (interaction with glucagon-binding sites and activation of adenylate cyclase) using gel filtration, ion-exchange and high-performance liquid chromatography. Depending on the criteria chosen, this peptide is referred to as either 'bioactive enteroglucagon' (activity in liver), 'oxyntomodulin' (specific action in gastric oxyntic glands) or 'glucagon-37' (chemical structure).

Glucagon-37 *Oxyntomodulin* *Bioactive enteroglucagon* *Isolation* *Porcine jejuno-ileum*

1. INTRODUCTION

The presence of glucagon-like material in intestine has been long known, owing to its interaction with glucagon-sensitive biological [1] and immunological [2] systems. Using in vitro models, we have shown that a porcine intestinal peptide that displays glucagon-like immunoreactivity was able to interact with the glucagon-receptors and to activate the adenylate cyclase present in liver membranes [3,4]. We have used these criteria for following up the different steps of purification of the peptide responsible for those activities. This paper describes the isolation procedure of the peptide which is referred to as 'bioactive enteroglucagon' because of its glucagon-like effect in liver, or 'oxyntomodulin' owing to its specific action on the acid-secreting gastric area [5]. The term 'glucagon-37' refers to its chemical structure, described in [6].

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2. MATERIALS AND METHODS

2.1. *Study of the binding of ^{125}I-glucagon to rat liver plasma membranes*

Rat liver plasma membranes were prepared according to step 11 in [7]. Porcine pancreatic glucagon was labeled with Na^{125}I at ~0.4 iodine atoms/molecule according to a modification [4] of the chloramine T procedure [8]. Binding studies were done as in [4].

2.2. *Study of the adenylate cyclase activity*

Adenylate cyclase activity and its stimulation by the different fractions were measured in rat liver plasma membranes as in [4] using a radioimmunoassay procedure for cyclic AMP [9].

2.3. *Extraction procedures*

Porcine intestines were extracted by the methods used for the isolation of several gastro-intestinal peptides [10]. The procedure includes a heat-coagulation of the bulk of the proteins, an extraction of polypeptides with 0.5 M CH_3COOH, adsorption on alginic acid, elution with 0.2 M HCl and precipitation with NaCl at saturation (see section 3).

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2.4. *Gel filtration procedures*

Sephadex G-25 gels (fine or coarse; Pharmacia, Uppsala) were prepared and packed in commercially available glass columns using the technique in [11]. Bio-Gel P6 100–200 mesh (Bio-Rad, CA) was prepared and packed according to the manufacturer. All gel filtrations were performed with 0.2 M CH_3COOH as the eluent and monitored by UV absorbance at 280 nm using Zeiss or Gilford instruments. The fractions were pooled and lyophilized (see figure legends).

2.5. *Ion-exchange chromatography*

CM-Sepharose (Pharmacia) was prepared according to the manufacturer. The chromatography was run in 22.5 mM pyrophosphate buffer (pH 6.5) containing 5% dimethylformamide and eluted with a linear gradient generated by a peristaltic pump (P3, Pharmacia) of 0–0.3 M NaCl in the same buffer at constant pH. The fractions were pooled, desalted by gel filtration on a Sephadex G-25 (coarse) 2.5 × 100 cm column eluted with 0.2 M CH_3COOH, and lyophilized.

2.6. *High-performance liquid chromatography (HPLC)*

The apparatus consisted of two model 6000 A solvent delivery systems controlled by a model 660 solvent programmer, a U6K injector, a model 440 UV detector operated at 280 nm and a model 450 variable wavelength detector (operated at 220 or 215 nm). These instruments were from Waters Assoc. (France). Peak heights and peak areas were calculated by a SP 4100 computing integrator (Spectra Physics, USA). The column (Zorbax ODS 0.46 × 25 cm, Du Pont de Nemours, USA)–solvent system (1% CF_3COOH buffered with diethylamine at pH 2.5) was that in [12]. The column used for desalting (μ Bondapak CN 0.4 × 30 cm) was from Waters Assoc.

2.7. *Flat bed isoelectrofocusing*

It was performed on G-75 (superfine) Sephadex gel from Pharmacia in a Desaga (FRG) apparatus with a LKB (Sweden) power delivery system and LKB ampholytes pH 3–10 according to [13].

2.8. *Peptides*

Porcine pancreatic glucagon (highly purified grade) from the Novo Res. Inst. (Copenhagen) was used without further purification for standard solutions and was repurified by HPLC followed by lyophilization before iodination.

2.9. *Chemicals*

CF_3COOH (TFA) was spectroscopic grade (Uvasol, Merck, Darmstadt) for the purification step. Sequence grade TFA from Pierce (USA) in 1 g ampules was used for the desalting step. Carrier-free $Na^{125}I$ (IMS 300) for iodination was from Amersham (England). Diethylamine 'analyzed reagent' was from Baker (Netherlands). Acetonitrile (CN_3CN) was spectroscopic grade (Uvasol), or pro analysi grade from Merck. All other chemicals were pro analysi grade. Water was purified by running tap water into mixed bed resins followed by a Milli-Q system (Millipore, USA).

3. RESULTS

3.1. *Preparation of the starting material*

The concentration of bioactive enteroglucagon (fig.1) measured according to its glucagon-like effect in rat liver is low in porcine duodenum (~0.45 μg/kg gut; i.e., 0.13 nmol/kg gut*), increases in the first meters of jejunum and fluctuates between 1.5–3 μg (0.43–0.86 nmol*)/kg gut along jejuno-ileum. However, there are no gross differences between the concentrations of the peptide in the first half and in the second half of por-

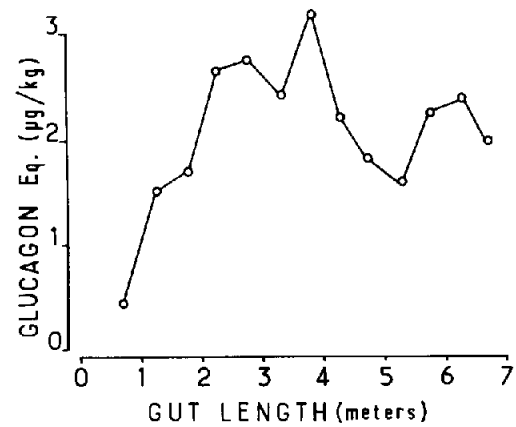

Fig.1. Concentration of bioactive enteroglucagon as measured by interaction with glucagon-receptors in rat liver membranes [4], as a function of the porcine small intestinal segment. Conditions for extraction and assay are indicated in section 2.

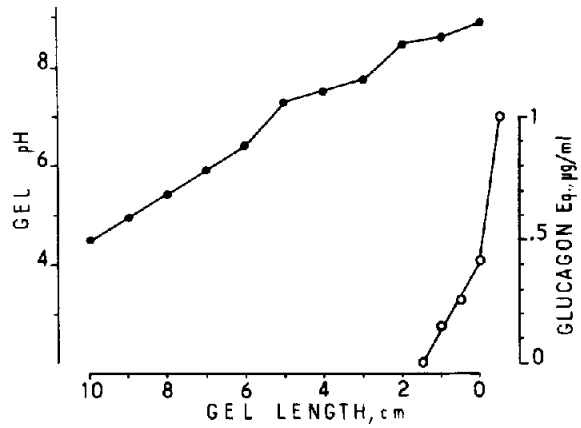

Fig.2. Analysis of crude extract of porcine jejuno-ileum (see section 3) by isoelectrofocusing on Sephadex gel [13]. The glucagon-like binding activity was measured in samples of the focusing gel at the end of the focusing experiment. Details may be found in section 2 and the references.

cine jejuno-ileum (2.35 µg or 0.67 nmol*/kg *vs* 2.1 µg or 0.6 nmol*/kg). Accordingly and so as to simplify collection on a large scale of the desirable

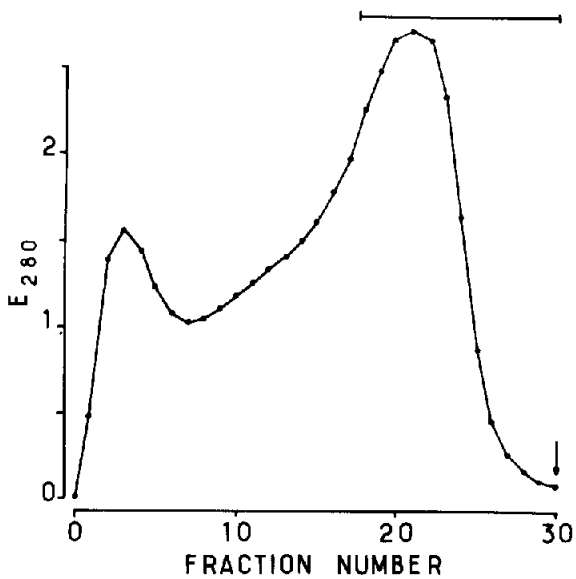

Fig.3. Gel filtration of crude porcine jejuno-ileum extract (salt-cake) [10] on a Sephadex G-25 (fine) 10 × 100 cm column: load, 17 g salt-cake in 170 ml 0.2 M CH_3COOH; flowrate, 2.9 l/h, 100 ml-fractions. The fraction indicated by the bar was pooled, lyophilized and used for further processing.

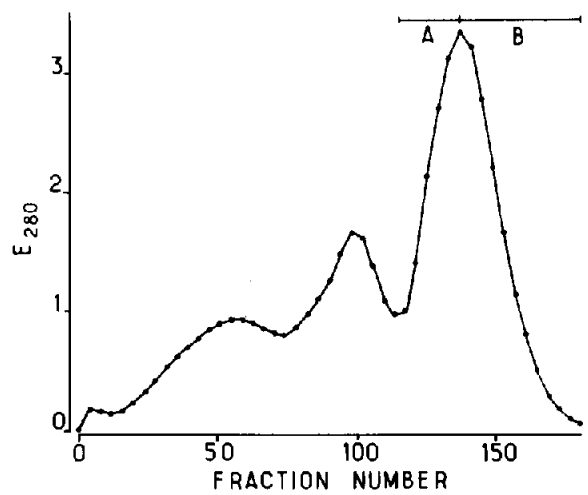

Fig.4. Gel filtration of the G-25 pool (fig.3) on a Bio-Gel P6 (100–200 mesh) 5 × 100 cm column: load, 2 g lyophilized powder in 30 ml 0.2 M CH_3COOH; flowrate 350 ml/h; pool A contained the bulk of VIP (see section 3), pool B was lyophilized and used for further processing.

part of intestine, the last 4–8 m (depending on the batch) of porcine jejuno-ileum were collected in a slaughter-house as soon as possible after the death of the animals and treated as in [10]. Briefly, gut was rinsed with tap water, boiled in water for 10 min, frozen, minced and extracted in 0.5 M CH_3COOH. The extracted peptides were adsorbed on alginic acid, eluted with 0.2 M HCl and precipitated with NaCl at saturation. The sequence of adsorption, elution and precipitation was repeated once. For analytical purpose, a small portion of this precipitate was desalted by gel filtration and run on flat bed isoelectrofocusing [13]. The apparent isoelectric point of bioactive enteroglucagon was >9 (fig.2). This indicates that, in agreement with its determined structure [6], this peptide is much more basic than pancreatic glucagon (isoelectric point ~6.5).

3.2. *Isolation procedure*

The peptide precipitate was subjected to gel fil-

* On a molar basis, the actual concentrations are ~5-times higher, owing to the lower affinity of bioactive enteroglucagon *vs* pancreatic glucagon for the hepatic glucagon-receptor [4]

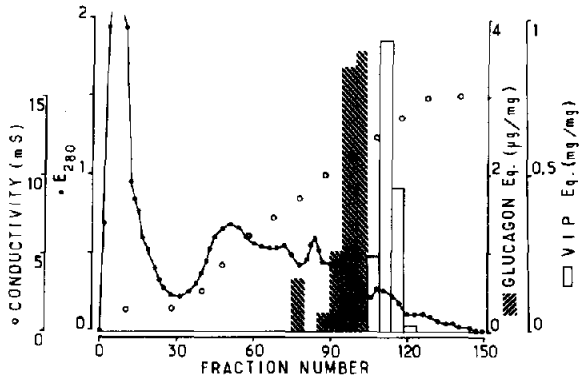

Fig.5. Cation exchange chromatography of pool B from Bio-Gel P6 (fig.4) on a CM-Sepharose 1.5 × 30 cm column. Details are given in sections 2 and 3: flowrate 77 ml/h, 6 ml fractions. Fractions 90–105 were pooled, desalted by gel filtration (section 2), lyophilized and used for further processing.

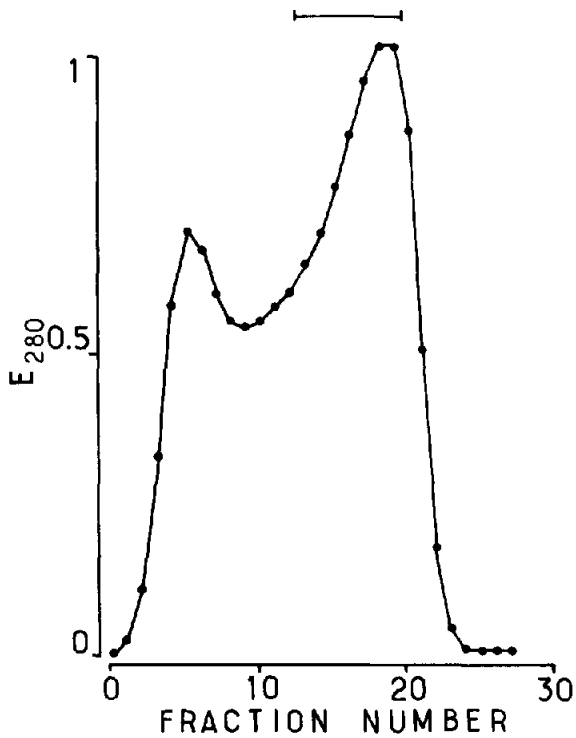

Fig.6. Gel filtration of the 90–105 pool from CM-Sepharose (fig.5) on a Sephadex G-25 (fine) 1 × 200 cm column: load, 40 mg lyophilized powder in 1 ml 0.2 M CH_3COOH, 2.5 ml fractions; flowrate, maintained at 16 ml/h by use of a peristaltic pump. The pool indicated by the bar was lyophilized and used for further processing.

tration on Sephadex G-25 (fig.3). The fraction indicated by the bar contained all the activity corresponding to bioactive enteroglucagon. This holds true for the other peptides of the family: secretin [14], vasoactive intestinal peptide (VIP) [11] and porcine intestinal peptide having N-terminal histidine and C-terminal isoleucine amide (PHI) [15]. After lyophilization, this fraction was further purified by gel filtration on Bio-Gel P6 (fig.4). The

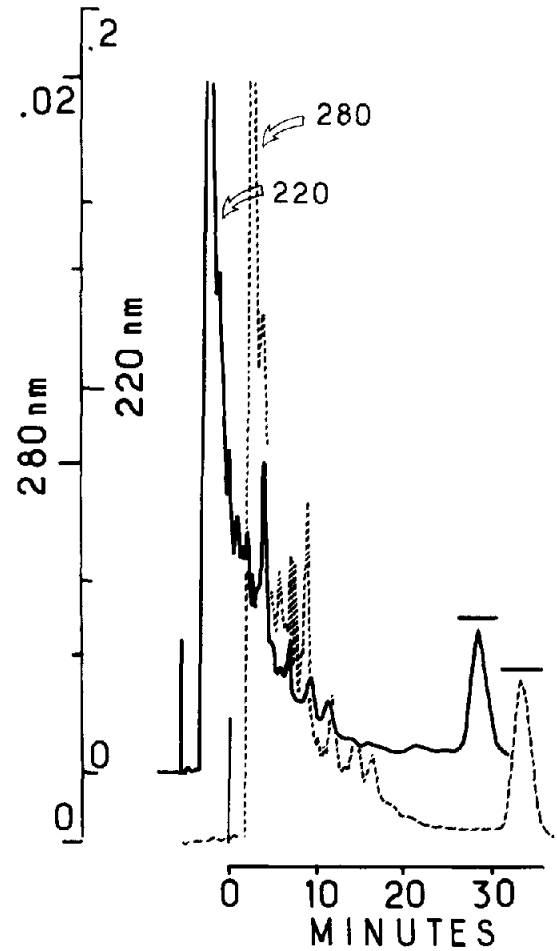

Fig.7. High-performance liquid chromatography (HPLC) of the pool from the G-25 column (fig.6) . The column–solvent system was that in [12]. Details are given in section 2. The separation was obtained under the following conditions: flowrate 1.5 ml/min; isocratic at 32% CH_3CN in 1% TFA/Diethylamine (pH 2.5); detection at both 220 nm (0.2 UAFS) and 280 nm (0.02 UAFS). The peak indicated by the bar was evaporated in vacuo and injected onto the desalting column (fig.8).

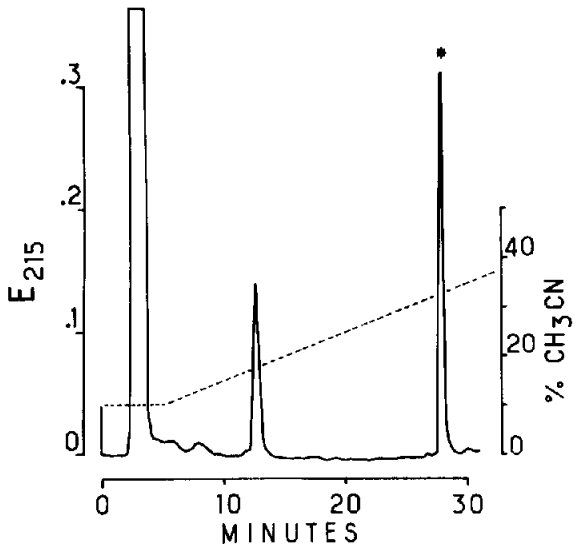

Fig.8. HPLC of the peak obtained as shown in fig.7. Conditions (section 2): column μCN 0.4 × 30 cm (Waters Assoc.); aqueous solvent 0.13% (w/w) TFA; organic solvent, CH_3CN containing 0.05% $CH_3COOC_2H_5$, 1.5 ml/min; linear gradient from 10–40% organic for 30 min. Detection at 215 mm 0.4 AUFS. The peak at 3 min represented the diethylamine trifluoroacetic salt from the preceding column (fig.7). The peak at 12 min, absent when spectroscopic grade CH_3CN was used for the isolation step (fig.7), represents a UV-absorbing impurity present in pro analysi grade CH_3CN. The peak at 29 min indicated by a star in the salt-free pure peptide that was lyophilized for characterization of its biological and chemical features [6].

most retarded fraction (B) contained bioactive enteroglucagon, whereas the bulk of VIP was present in fraction A (fig.4).

After lyophilization, fraction B was chromatographed on a cation-exchange column eluted with a linear gradient of ionic strength (fig.5). Pools of the fractions were desalted by gel filtration. The activity eluted as 2 discrete peaks. The first one, the nature of which is unknown, was constantly observed in the numerous column runs. The bulk of the activity eluted in front of VIP (fig.5). On the basis of its glucagon-like immunoreactivity (GLI) content, as measured with an antibody that recognizes similarly the glucagon-related peptides of either pancreatic or intestinal origin, this peak was ~20% as potent as pancreatic glucagon on the binding of ^{125}I-glucagon to its hepatic receptors and ~10% as potent as pancreatic glucagon in stimulating hepatic adenylate cyclase. These characteristics are those of the peptide suspected to be present in the crude extracts [3,4,16] and which were chosen as the criteria for following up the purification procedure. Accordingly, this fraction was selected for further processing.

Table 1

Purification factors and yields obtained at each step of the isolation procedure

Step	Factor	Yield
G-25	6	60%
P-6	5	50%
CM	15	50%
G-25	5	60%
HPLC-1	40	80%
HPLC-2		100%
Total	90 000	7%

Purification factors were calculated from the increase in specific activity of the lyophilized powders as measured by the binding assay. The yields were calculated from the total activity recovered divided by that recovered in the preceding step

After another gel filtration step (fig.6), the peptide was isolated by HPLC (fig.7) using the column–solvent system developed in [12]. This step allowed, with a 40-fold purification factor, separation of bioactive enteroglucagon from the foreign peptidic material (fig.7). Another HPLC run using completely volatile solvents (fig.8) yielded, after lyophilization, the peptide in a salt-free pure form.

Table 1 summarizes the different steps of purification; with an overall yield of ~7%, one ton (boiled wet wt) of porcine jejuno-ileum yielded ~0.5 mg pure peptide.

4. DISCUSSION

In 1948, Sutherland et al. [1] observed that, similarly to pancreatic glucagon, intestinal extracts were able to activate the hepatic adenylate cyclase. However, they did not further characterize the material showing this activity. On the other hand, the development by Unger's group of the glucagon radioimmunoassay allowed investigation of the GLI present in different tissues from several species. Of

particular interest was the study by Valverde et al. [2] on the characterization of GLI in dog intestine: on gel filtration, mainly 2 peaks of GLI were found, a prominent one of relatively high M_r (8000–9000) and a smaller one, eluting not far from pancreatic glucagon. In 1973–1974 [3,4] we showed, using the methods developed for the study of glucagon-receptors [17], that the small, low-M_r second peak contained a molecule that was able to bind to the hepatic glucagon-receptor and to activate, presumably through this receptor, hepatic adenylate cyclase. Using these in vitro characteristics for screening the fractions during the purification procedure, we succeeded in isolating the peptide responsible for these activities.

If we disregard the minor peak observed during the ion-exchange chromatography (fig.5) which may represent a degraded (e.g., deamidated) form of the active peptide, a single peak of activity on hepatic receptors has been observed all along the purification. This homogeneity leads us to conclude that the peptide that we have isolated is the main, if not the single, biologically active peptide present in porcine intestine that interacts with the glucagon-sensitive hepatic system. Accordingly, this peptide is likely to represent the intestinal hyperglycemic–glycogenolytic factor discovered in [1]; the term 'bioactive enteroglucagon' appears thus to be convenient. On the other hand, the term 'oxyntomodulin' describes in a more adequate way the peptide in terms of biological activity since its main action appears to be at the level of the acid-secreting oxyntic glands of stomach [5] rather than at the hepatic level; 'glucagon-37' is related to its chemical structure [6].

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