

# Purification of bovine pancreatic glucagon as a by-product of insulin production

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## Abstract

A process for purifying bovine pancreatic glucagon as a by-product of insulin production is described. The glucagon-containing supernatant from the alkaline crystallization of insulin was precipitated using ammonium sulfate and isoelectric precipitation. The isoelectric precipitate containing glucagon was then purified by ion-exchange chromatography on Q-Sepharose FF, gel filtration on Sephadex G-25 and ion-exchange chromatography on S-Sepharose FF. A pilot scale test was performed with a recovery of 87.6% and a purification factor of 8.78 for the first chromatographic step, a recovery of 75.1% and a purification factor of 3.90 for the second, and a recovery of 76.2% and a purification factor of 2.36 for the last one. The overall yield was 50%, a purification factor of 80.8 was obtained and the fraction containing active glucagon (suitable for pharmaceutical preparations) was 84% pure as analyzed by HPLC.

## Key words

- Glucagon
- Protein purification
- Gel filtration
- Ion-exchange chromatography
- Insulin

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## Introduction

Bovine pancreatic glucagon is a peptide secreted by the alpha cells of Langerhans islets which contains 29 amino acids and has a molecular mass of 3,485 Da calculated from its primary structure. Bovine glucagon has the same amino acid composition (1) as human (2) and porcine glucagon (3).

Pancreatic glucagon plays an important role in the maintenance of blood glucose concentration at normal levels and possibly also of free fatty acids and amino acids. Other related functions of glucagon include insulin release and regulation of protein catabolism. In addition, the hormone plays a role in the maintenance of normal cardiac function, regulation of urinary electrolyte

excretion, renal and hepatic blood flow, appetite regulation, gastric secretion and gut motility.

Glucagon is used as a drug mainly in cases of hypoglycemia in insulin-dependent diabetics (4,5), following insulin administration. Since prolonged hypoglycemic conditions may produce cortical injury, glucagon must be given in order to restore blood glucose to normal levels. Glucagon has also been used in the treatment of hypodynamic heart disorders (6) and hypotonic intestine (7).

Staub et al. (8) succeeded in isolating and crystallizing glucagon from an amorphous fraction obtained during the commercial purification of insulin. They employed repeated precipitation culminating in crystallization which was in itself a purification step. Re-

cently glucagon separation has been based more directly on molecular size and charge. Pollock and Kimmel (9) used ion-exchange chromatography on CM-Sephadex and QAE-Sephadex. Cole (10) used cation-exchange chromatography and buffers containing urea which is particularly beneficial since it not only permits chromatographic purification at pH values where glucagon has low solubility, but also helps to reduce protein self-association. Sundby and Markussen (11) developed a procedure applicable only to small-scale purification using the mother liquor fraction from the citrate crystallization of insulin brought to about half-saturation with NaCl. Glucagon was obtained from the precipitate by gel filtration and isoelectric focusing.

The present paper describes a procedure for the purification of bovine glucagon using a starting material similar to that used by Sundby and Markussen (11). The glucagon in the mother liquor fraction from the alkaline crystallization of insulin was isolated by salting-out with ammonium sulfate and isoelectric precipitation.

The isoelectric precipitate was purified by ion-exchange chromatography on Q-Sepharose FF in buffer containing urea, by gel filtration on Sephadex G-25, and by ion-exchange chromatography on S-Sepharose FF. The process can be used for large-scale purification of glucagon (470 mg isoelectric precipitate containing 225 mg glucagon as analyzed by radioimmunoassay (RIA), applied to a 45-l Q-Sepharose column) and the hormone obtained has sufficient purity to be used in pharmaceutical formulations.

## Material and Methods

### Radioimmunoassay

The analysis was performed as described by Christofides (12).  $^{125}\text{I}$ -labeled glucagon obtained by the method of Bryant (13) was purified from free  $^{125}\text{I}$  and other contami-

nants by high performance liquid chromatography (HPLC) using a Nova Pak  $\text{C}_{18}$  column (Waters, Milford, MA) and an acetonitrile gradient in 0.1% trifluoroacetic acid. The fractions were collected into tubes containing PBS buffer. Glucagon antibodies and glucagon for the standard curves were obtained from Dr. Stephen R. Bloom (Royal Post Graduate Medical School, London). Glucagon for  $^{125}\text{I}$  labeling was obtained from Peninsula Laboratories (Belmont, CA).

### High performance liquid chromatography

The analyses were performed at 45°C using a Pharmacia-LKB Biotechnology HPLC system consisting of two pumps (model 2152), a variable wavelength monitor (model 2151), an integrator recorder (model 2221) and a 4.6 x 250-mm Aquapore RP 300 column (Brownlee, Foster City, CA). A stepwise acetonitrile gradient consisting of 50, 60, 62, 70 and 80% buffer "B" (5 min for each concentration) was used (buffer "A", 50 mM sodium phosphate, 10 mM perchloric acid, pH 2.5, and buffer "B", 50% acetonitrile in buffer "A"). The flow rate was 1 ml/min and the elution was monitored by absorbance at 214 nm. Commercial glucagon (Lilly) was used as a standard.

### Protein analysis

Protein concentration was determined according to Lowry et al. (14) using BSA as standard.

### Ammonium sulfate and isoelectric precipitation

Proteases present in the pancreatic extracts were precipitated with ammonium sulfate (commercial grade) according to Kunitz (15). The pH of the mother liquor of alkaline insulin crystals was adjusted to 2.5-3.0 with 3 N HCl. An amount of 452 ml of 95% ethanol and 4.0 ml of 20% zinc chloride was

added (at a temperature of less than 20°C) per liter of solution. The pH was adjusted to 2.1-2.2 with 3 N HCl and 14 ml of 50% ammonium sulfate solution per liter of mother liquor solution was added slowly. The pH was adjusted to 2.2-2.4 (20-30°C), the suspension was stirred for 2 h and then left to stand unstirred for 12 h and filtered.

Glucagon in the supernatant was precipitated by adjusting the pH to its isoelectric point (pH 7.0) with 3 N HCl. After 24 h at 4°C glucagon was collected by filtration in a plate and frame filter press. Glucagon concentration was 10.5 mg/g isoelectric precipitate, as determined by RIA.

#### **Ion-exchange chromatography on Q-Sepharose FF**

The isoelectric precipitate was purified using a Q-Sepharose FF column (2.0 x 26 cm) from Pharmacia-LKB Biotechnology (Uppsala, Sweden). The column was eluted at room temperature with 50 mM Tris-HCl, 7 M urea, pH 9.0, at a flow rate of 5.0 ml/min. Before utilization, urea (commercial grade) solutions were purified by deionization in Amberlite MB-20 to 5 µS conductivity. Forty-ml samples (400 mg of isoelectric precipitate) were applied and eluted with a stepwise NaCl gradient. Fractions of 5 ml were collected, absorbance at 280 nm was measured and glucagon was identified by RIA.

#### **Gel filtration chromatography on Sephadex G-25**

The glucagon fraction (70 ml) from Q-Sepharose FF was applied to a Sephadex G-25 (Pharmacia, LKB Biotechnology) column (3 x 100 cm) and eluted at room temperature with 0.1 M acetic acid at a flow rate of 7 ml/min. Fractions of 10 ml were collected, absorbance was measured at 280 nm and glucagon was determined by HPLC. The glucagon-rich fractions were pooled and lyophilized.

#### **Ion-exchange chromatography on S-Sepharose FF**

One hundred mg of the glucagon fraction obtained in the previous step was applied to an S-Sepharose FF (Pharmacia, LKB Biotechnology) column (1.6 x 10 cm). The sample (4 ml) containing 100 mg of glucagon was applied to the column and eluted with 50 mM sodium citrate buffer, 7 M urea, pH 4.0, at a flow rate of 2.5 ml/min. Fractions of 3 ml were collected, absorbance was measured at 280 nm and glucagon was determined by HPLC analysis.

#### **Pilot scale test**

The same procedure was developed at the pilot scale using the following chromatographic conditions: 1) the isoelectric precipitate dissolved in 1 liter was applied to a 37 x 45-cm Q-Sepharose FF column and eluted at a flow rate of 750 ml/min; 2) 5.25 ml of the glucagon fraction from Q-Sepharose FF was applied to a 25 x 120-cm Sephadex G-25 column and eluted at a flow rate of 450 ml/min; 3) 100 ml of the glucagon fraction from the previous step was applied to a 4.5 x 25-cm S-Sepharose FF column and eluted at a flow rate of 10 ml/min.

#### **Bioassay**

The hyperglycemic glucagon activity was tested after subcutaneous injection of the samples into rats, according to Staub et al. (8). Blood glucose concentration was determined by the method of Barhan and Trinder (16).

#### **Results**

The starting material selected for glucagon purification was the mother liquor from the alkaline insulin crystallization obtained in the industrial production of bovine insulin. This material is the major source of pancreatic glucagon (11).

Figure 1 - Ion-exchange chromatography of glucagon on Q-Sepharose FF. The isoelectric precipitate (400 mg/ml eluent) was applied to a 2.0 x 26-cm column and eluted at room temperature with 50 mM Tris-HCl, 7 M urea, pH 9.0, at a flow rate of 5.0 ml/min. Fractions of 5.0 ml were collected and the amount of glucagon was determined by RIA. The arrow indicates the fraction where the buffer NaCl concentration was increased to 1.0 M NaCl. Absorbance at 280 nm (solid line), mg glucagon (dashed line).

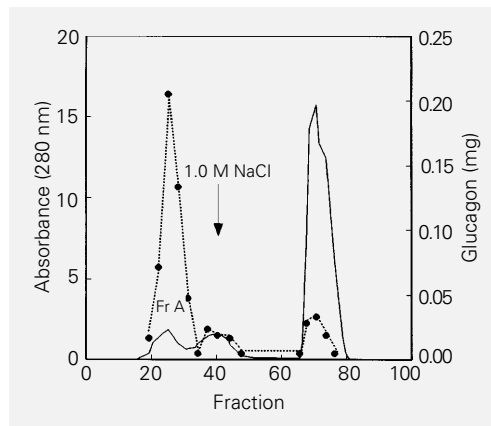
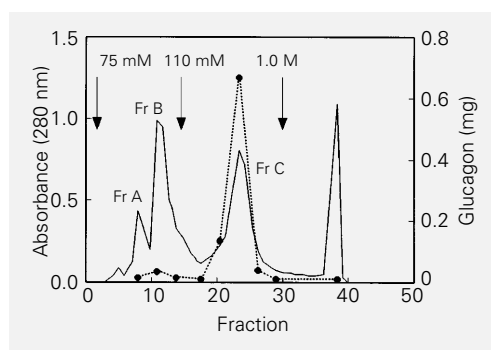


Figure 3 - Ion-exchange chromatography of glucagon on S-Sepharose FF. The sample, 100 mg of fraction B (Fr B) from Sephadex G-25 (Figure 2), was applied to a 1.6 x 10-cm column and eluted at room temperature with 50 mM sodium citrate, 7 M urea, pH 4.0, at a flow rate of 2.5 ml/min. Fractions of 3.0 ml were collected and the amount of glucagon was determined by HPLC. The arrows indicate the fractions where the buffer NaCl concentration was increased by the addition of NaCl. Absorbance at 280 nm (solid line), mg glucagon (dashed line).



The first step of the purification process was salting-out of proteases usually present in pancreatic extracts with ammonium sulfate. Glucagon was concentrated by adjusting the pH to its isoelectric point, resulting in a precipitate with 1.04% (w/w) glucagon when analyzed by RIA. The isoelectric precipitate was dissolved and applied to a Q-Sepharose FF column (Figure 1). Glucagon was eluted mainly in the first peak, as can be seen from the radioimmunoassay data. Glucagon recovery in this step was 80 to 88% with a purification factor of 8.2 to 8.9. The glucagon fraction from Q-Sepharose FF was desalted on a Sephadex G-25 column (Figure 2). Higher molecular weight proteins were also removed, resulting in a purification factor of 3.5 to 4.0. The recovery in this step was 74-82%. The fractions containing glucagon, as indicated by HPLC, were pooled and further purified by ion-exchange chromatography on S-Sepharose FF (Figure 3).

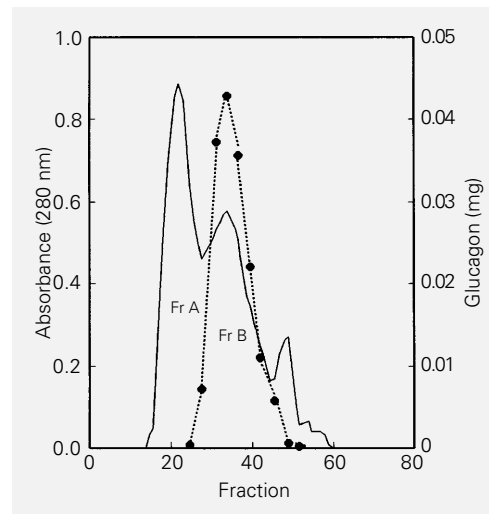


Figure 2 - Gel filtration chromatography of glucagon on Sephadex G-25. The sample, 70 ml of fraction A (Fr A) from Q-Sepharose FF (Figure 1), was applied to a 3.0 x 100-cm column and eluted at room temperature with 0.1 M acetic acid at a flow rate of 7.0 ml/min. Fractions of 10 ml were collected and the amount of glucagon was determined by HPLC as shown in Figure 4. Absorbance at 280 nm (solid line), mg glucagon (dashed line).

The recovery was 76-79% and a purification factor of 2.3 to 2.5 was obtained. The fractions containing glucagon, as indicated by HPLC, were pooled, dialyzed and lyophilized. Figure 4 shows the standard and purified glucagon HPLC analysis after the final purification step.

This procedure was utilized at the pilot scale and the results obtained in the different steps are shown in Table 1. The total recovery was 50.2% and a purification factor of 80.8 was obtained.

To measure the hyperglycemic action, purified and standard (commercial Lilly) glucagon were injected into rats. The results in Figure 5 show that glucagon obtained by the present process induced hyperglycemia at levels similar to those of standard glucagon.

## Discussion

Glucagon can be produced synthetically

(17,18) but since the primary structures of porcine, bovine and human glucagon are identical, synthetic glucagon has no advantage over glucagon of bovine or porcine origin. Since the need for glucagon has not exceeded the amount that can be produced as a by-product of insulin, glucagon for clinical use will probably continue to be produced from pancreas.

The process described here for glucagon purification provides a recovery of 50% which is the same or higher when compared with other processes such as those used by Maskalick and Anderson (19), Jackson (20) and Stilz and Jackson (21), with recoveries of 44.6, 33.2 and 43.5%, respectively. Among these investigators, only Jackson (20) isolated glucagon from insulin crystal mother liquors.

In addition to obtaining glucagon as a by-product of industrial insulin production, the process presented here has an additional advantage of using chromatographic steps, mainly ion-exchange chromatography, which can be easily scaled up as documented in Table 1.

The 84% pure glucagon (HPLC analysis), which has also been characterized by amino acid analysis and protein sequencing (both of them performed at the Laboratório de Bioquímica e Química de Proteínas, Universidade de Brasília), has the necessary quality for use in pharmaceutical formulations. Furthermore, the HPLC procedure can be used to prepare at least 90% (HPLC analysis) pure glucagon on an industrial scale (22). Finally, biological assay of glucagon obtained by this process showed its capacity to induce hyperglycemia at levels similar to those obtained with a standard glucagon preparation.

Table 1 - Pilot scale chromatographic production of glucagon.

\*Determined by the method of Lowry et al. (14). % Glucagon was determined by RIA (a) or HPLC (b). The total yield was 50%.

Purification step	Protein* (g)	Glucagon (g)	Purification (x)	Yield (%)
Isoelectric precipitation	225.0	2.34 (a)	-	-
Q-Sepharose FF	22.50	2.05 (a)	8.78	87.6
Sephadex G-25	4.33	1.54 (b)	3.90	75.1
S-Sepharose FF	1.397	1.174 (b)	2.36	76.2

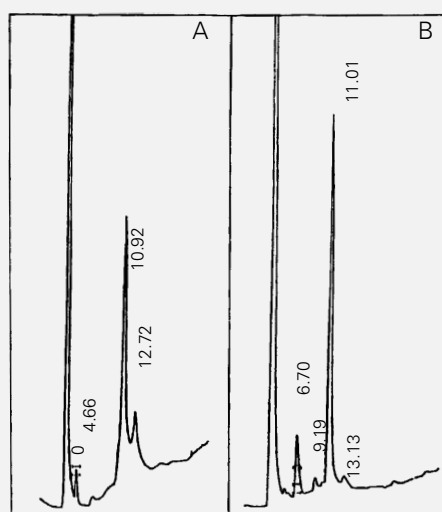


Figure 4 - Analytical characterization of glucagon by HPLC. A, Purified glucagon (Fr C from S-Sepharose FF). B, Standard glucagon. Purified and standard glucagon have the same retention time ( $10.97 \pm 0.08$ ) and approximately the same purity (84.05 and 85.2% for purified and standard glucagon, respectively). Experimental conditions: after sample application, a stepwise acetonitrile gradient consisting of 50, 60, 62, 70 and 80% buffer "B" (5 min per step) was used (buffer "A", 50 mM sodium phosphate, 10 mM perchloric acid, pH 2.5, and buffer "B", 50% acetonitrile in buffer "A") in an Aquapore RP 300 column (4.6 x 250 mm) at room temperature and at a flow rate of 1 ml/min. The elution was monitored by absorbance at 214 nm.

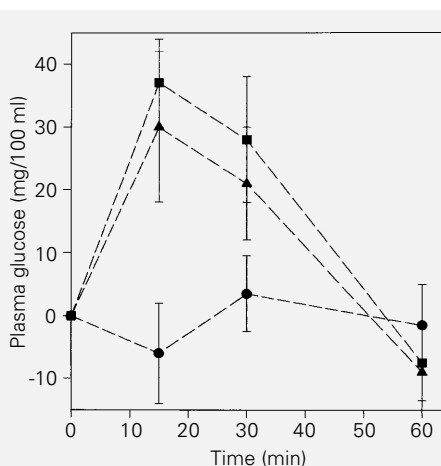


Figure 5 - Hyperglycemic action of glucagon on mice. Circles, Control (without glucagon); triangles, purified glucagon (Fr C from S-Sepharose FF); squares, standard glucagon. The mice (fasted females weighing 18 to 25 g) were divided into three groups (8 mice per group), which received by subcutaneous injection 2.5  $\mu$ g of purified glucagon (Fr C from S-Sepharose), standard glucagon or saline solution. Forty- $\mu$ l blood samples were taken and centrifuged and plasma glucose was determined by the method of Barhan and Trinder (16). Each point represents the means of six determinations.

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